PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
C12Q 1/00, G01N 33/53	A1	WO 93/04191
		(43) International Publication Date: 4 March 1993 (04.03.93)

(21) International Application Number: PCT/US92/06823

(22) International Filing Date: 13 August 1992 (13.08.92)

(30) Priority data:
745,158 15 August 1991 (15.08.91) US

(71) Applicant: NEORX CORPORATION [US/US]; 410 West Harrison, Seattle, WA 98119 (US).

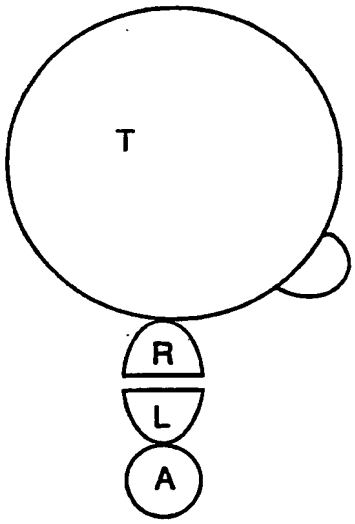
(72) Inventors: MORGAN, Alton, Charles, Jr. ; 20102 Cedar Valley Road, Suite 205, Lynnwood, WA 98036 (US). ABRAMS, Paul, G. ; 2125 First Avenue, Seattle, WA 98121 (US).

(74) Agent: LEVINE, Michael, L.; Stoel Rives Boley Jones & Grey, 900 SW Fifth Avenue, Suite 2300, Portland, OR 97204 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: NONCYTOLYTIC TOXIN CONJUGATES



(57) Abstract

The present invention provides conjugates useful for modifying target cell functions to achieve therapeutic results. Conjugates of the present invention may include a noncytolytic toxin that does not directly inhibit protein synthesis and is capable of operating through an existing cellular metabolism signaling mechanism; and a targeting moiety that constitutes a ligand recognized by the target cell receptor involved in that existing signaling mechanism. Alternatively, conjugates of the present invention may include a toxin domain capable of directly impacting a target cell metabolic process (e.g., catalyzing conversion of ATP to cAMP) or acting on a substrate that is implicated in such a pr cess (e.g., actin); and a targeting moiety exhibiting specificity for the target cell population. Methods of using these conjugates are also discussed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland			SE	Sweden
CI	Côte d'Ivoire	KR	Republic of Korea	SK	Slovak Republic
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CS	Czechoslovakia	LK	Sri Lanka	SU	Soviet Union
CZ	Czech Republic	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America

NON-CYTOLYTIC TOXIN CONJUGATES

Technical Field

The present invention relates to therapeutic conjugates useful in activating, suppressing, or in some way altering a biological response in target cells. Such conjugates include a proteinaceous non-cytolytic toxin moiety conjugated by chemical or recombinant means to a targeting moiety.

Background of the Invention

Many proteinaceous biological response modifiers (BRMs) have been identified, isolated, and cloned from eukaryotic sources, and used in a variety of clinical applications over the past decade. Exemplary BRMs obtained from eukaryotic cell sources, the cell source, and the target cells for such BRMs are presented in Table I.

TABLE I

BRM	SOURCE	TARGET
IL-1	Macrophages, activated T-cells. Many other lymphoid and non-lymphoid cells.	Most endstage and progenitor lymphoid cells.
IL-2	Activated T-cells.	B-cells, T-cells, monocytes, and NK cells
IL-3	T-cells.	Early progenitor cells, mast cells.
IL-4	Activated T-cells.	T-cells, B-cells.
IL-5	T-cells.	B-cells, eosinophils.
IL-6	Macrophages, activated T-cells, and other non-lymphoid cells.	B-cells, hepatocytes.
IL-7	Activated T-cells.	Early lymphoid progenitors, activated B-cells.
IL-8	Activated T-cells, Endothelial cells.	PMNs.
GM-CSF	Activated T-cells.	Granulocytic and myeloid endstage and progenitor cells, T-cells, monocytes.
G-CSF	Activated T-cells.	Granulocytic endstage and progenitor cells.
M-CSF or CSF-1	Macrophages.	Monocytic endstage and progenitor cells.

As the literature on eukaryotic cell-derived BRMs has grown, common elements in the molecular mechanisms of action and in the regulation of BRMs as well as evolutionary relationships between the varied BRMs have become apparent.

The mechanism of action of BRMs varies according to the particular BRM and target cell involved. For instance, studies with GM-CSF indicate that a second messenger, cAMP, is involved in

mediating the priming of monocytes for enhanced antigen presentation. See Coleman et al., J. Immunol., 143:4134, 1989. In contrast, no role for cAMP has been demonstrated in GM-CSF activation of polymorphonuclear leukocytes for oxidative burst. See Di Persio et al., J. Immunol., 140:4315, 1988.

Additionally, one BRM-induced activity may be mediated through an effector molecule such as tyrosine kinase while another BRM-induced activity occurring within the same target cell may not. This phenomenon was evidenced with IL-2 in Mills et al., Cell, 55:91, 1988. The key to the induction of a target cell response to a given BRM appears to be the presence of a specific receptor on the target cell.

BRM activity is regulated in a variety of ways. Both positive and negative regulators of BRM activity exist. Molecules such as GM-CSF, G-CSF, IL-3 and EPO that stimulate proliferation of bone marrow progenitor cells have been known for some time, but inhibitors of proliferation also exist. See Lenfant et al., Proc. Nat'l. Acad. Sci. (USA), 86:779, 1989 and Del Rizzo et al., Exp. Hematol., 18:138, 1990. High levels of synthesis of such proliferation inhibitors are induced upon binding of the positive regulator (e.g., G-CSF) and lead to a dampening of the positive signal (i.e., proliferation) and the return of progenitor cells to the quiescent state. Such inhibitors may also be produced constititively in low amounts and participate in maintaining only a small proportion of progenitor cells in an active state of proliferation.

Duration of target cell response is dependent upon a number of factors including the serum half-life of the response-inducing BRM. See Cheever et al., J. Immunol., 134:3895, 1985 and Metcalf et al., Proc. Nat'l Acad. Sci. (USA), 85:3160, 1988. Conse-

quently, serum half-life can be modulated to increase in vivo BRM activity. BRMs with longer serum half-lives are being developed for therapeutic applications to reduce the requirement for continuous infusion of the BRM and to prolong BRM activity in vivo. See Knauf et al., J. Biol. Chem., 263:15064, 1988 and Takakura et al., Pharm. Res., 7:339, 1990. Serum half-life of a BRM is a function of a number of factors, such as 1) receptor mediated uptake of BRMs, usually evidenced by accumulation in hematopoietic or RES (i.e., reticuloendothelial system) organs; 2) non-specific uptake of BRMs, a function of BRM hydrophobicity and charge; 3) size of BRMs, since proteins larger than albumin generally have longer serum half-lives than proteins smaller than albumin due to lower rates of kidney filtration and excretion; 4) rate of metabolism and excretion of BRMs; and 5) binding or interaction of BRMs with serum glycoproteins or soluble receptors.

Several BRMs modulate the migration of leukocytes to sites of inflammation or leukocyte egress into the circulation. The arrival of leukocytes at inflammatory sites provides additional cellular sources for cytokine BRM stimulation and amplification. See Wang et al., Blood, 72:1456, 1988.

As previously stated, eukaryotic cell BRM activity appears to be mediated through cellular receptors. These receptors may vary in size, glycosylation, or surface expression, possibly even reflecting individual cellular differences in post-translational modification of the same gene product. For example, comparative studies with characterized CSF (i.e., colony stimulating factor) receptors from different cell types indicate that the receptor may differ biochemically from cell type to cell type

(Baldwin et al., Blood, 73:1033, 1989) even though these receptors may be related evolutionarily. See Durum et al., Immunol. Today, 11:103, 1990. Biochemical differences in receptors may lead to functional changes in receptor expression or affinity. This heterogeneity, together with differences in receptor coupling to effector molecules within the cell, results in a wide diversity in response to BRM binding to target cells of the same or different cell type. For example, binding of IL-2 to its receptor on T-cells leads to proliferation and activation; and binding of IL-2 to its receptor on monocytes leads to cytokine synthesis but not proliferation.

Also, BRM binding or uptake into tissues need not lead to any significant response. This has been recognized from bio-distribution studies with labeled eukaryotic BRMs. BRMs typically show high uptake into RES organs such as the liver and spleen as well as uptake into tissues not associated with a biological response. This latter uptake may reflect binding to non-functional receptors or non-specific uptake. In addition, tissues to which BRMs bind that are not associated with a BRM response may well exhibit such a response, with the cellular activity escaping the notice of investigators because that activity is not of sufficient magnitude or being investigated.

Also potentially modulating cellular response to BRMs is the interaction of those BRMs with soluble serum receptors, glycoproteins, or antibodies. Soluble receptors or binding proteins have been characterized from human urine or serum for a variety of cytokines. It is likely therefore that most cytokines undergo some interaction with serum constituents. Alpha-2-macroglobulin is a serum

protein known to interact with multiple cytokines. The effect of this serum protein-BRM interaction is unknown, but such interactions may serve to prolong the serum half-life of BRMs. Recent findings also indicate that antibodies to cytokines exist in normal sera. The effect of such antibodies on cytokine function is unknown, but the anti-BRM antibody appears to bind with low affinity to the BRM and not block the induction of a target cell biological response. Such a bound BRM appears to retain its BRM function and may exhibit a prolonged serum half-life.

There are a variety of interrelationships between eukaryotic BRMs and their receptors and cellular responses generated by binding of the BRM. Cytokine synthesis, for example, has been shown to be induced in a cascading fashion after binding of cytokines to their receptors, probably through activation of oncogenes and DNA binding proteins or through activation of second messengers. See Farrar et al., CRC Critical Reviews in Therapeutic Drug Carrier Systems, 5:229, 1989. For instance, BRM-stimulated IL-1 secretion by monocytes leads to the induction of tandemly regulated genes and coordinated synthesis of IL-2, IL-4, and GM-CSF in T-cells. See Oppenheim et al., Immunol. Letters, 16:179, 1987. Mitogenic stimulation of T-cells can lead to coordinated synthesis of TNF and gamma-interferon. See McBride et al., Cancer Res., 50:2949, 1990. As a result, a given biological response can be directly attributed to binding of a cytokine to its receptor on the target cell or can arise indirectly through cytokine interaction with another cell type that then secretes or expresses one or more lymphokines, inducing a pleiotropic target cell or tissue response. Consequently, some of the biological

activities of IL-2 and its usefulness in cancer therapy may result from its direct stimulation of sensitized T-cells (Mule et al., J. Immunol., 139:285, 1987) while much of its associated toxicity may result from its induction of TNF which leads to secondary toxicities. See Mier et al., J. Clin. Immunol., 8:426, 1988.

BRM activity has also been associated with bacterial cell products. For example, IL-1 has a spectrum of biological activities very similar to endotoxin, a cell wall constituent of gram negative bacteria. In addition, a number of bacterial compounds have been shown to have multiple BRM activities, including colony stimulating and IL-2-inducing activity. These activities have usually been associated with crude bacterial preparations, however.

As shown in Table II, bacterial enterotoxins or exotoxins are also a rich source of BRM activity.

TABLE II

TOXIN	TARGET CELL	BRM ACTIVITY
Pertussis	T-Cell	Proliferative
	T-Cell	Activation
	PMN	Activation
	Granulocytic Progenitor	Proliferative
	Mast Cell	Histamine Sensitization
Cholera	T-Cell	Inhibits Activation
	Early Progenitors	Inhibits Proliferation
Pseudomonas Exotoxin A	T-Cell	Activation
	Monocyte	Primes for Oxidative Burst
TSST-1		
SEB	T-Cell	Proliferation/
SEA	B-Cell (indirect)	Activation

Gelonin PAP	Monocyte, possibly	Suppression of Humoral and Cellular Responses

Most studies investigating toxin BRM activity have been carried out with native, whole toxins. In a small number of studies, a given BRM activity has been associated either with the enzymatic (i.e., catalytic) activity of the toxin or with its cell binding domain. These associations have not always been made unambiguously, however. For instance, lympho-proliferative activity of pertussis toxin has been associated with the toxin's enzymatic activity in one study, but with the B subunit or cell binding domain of the toxin in a different study.

Bacterial toxin BRMs share certain characteristics with their eukaryotic cell BRM counterparts. Both types of BRMs are soluble molecules and primarily induce effects in the region of the target cell but are capable of producing more distant effects. BRMs from bacterial toxins, however, differ markedly from eukaryotic BRMs in that the bacterial toxin BRMs exhibit an enzymatic (i.e., toxic) activity associated with a particular structural domain or subunit.

As can be seen from even a cursory examination of Tables I and II, BRMs may be useful in a wide variety of therapeutic applications. The ability of BRMs to impact many aspects of the immune system make such molecules attractive in the treatment of cancer, autoimmune deficiencies, and other conditions impacting the immune system.

A major goal for cancer therapy, for example, has been to improve the selectivity of otherwise toxic therapies by taking advantage of certain attributes of tumor cells that are distinctive from most normal cells. The degree of selectivity of a

therapeutic agent is usually assessed by its therapeutic index, i.e., the ratio of the potency of the therapeutic agent against a tumor to the toxicity of the agent with respect to normal cells. Consequently, cancer therapy can be improved by one or more of the following: (1) increasing the potency of the therapeutic agent against the tumor while not changing toxicity to normal organs; (2) decreasing the toxicity of the therapeutic agent to dose limiting normal organs; (3) improving the selectivity of the therapeutic agent for tumor cells over normal cells by targeting of the cytotoxic agent; or (4) delivering a molecule having effects on tumor cells (e.g., differentiation) that yield salutary results (i.e., slowing or cessation of growth or metastasis), with those same effects on normal cells (e.g., differentiation) not being deleterious. Of course, these approaches to improve therapeutic efficacy also apply to non-cancer therapeutic applications as well.

In order to improve the therapeutic index according to number 3 above, numerous investigators have utilized conjugates including a cytotoxic moiety and a targeting moiety selective for tumor cells. Toward this end, a variety of agents including radioisotopes, drugs, and toxins have been targeted by antibodies, growth factors, or hormones.

Protein toxins, for example, have been employed as toxic moieties in prior art conjugates referred to as immunotoxins. All of the toxins heretofore used in immunotoxin construction affect protein synthesis within the cell. Intact toxins or toxin portions used heretofore include diphtheria, Pseudomonas exotoxin A, shigella, ricin and abrin (plant). Plant toxins also occur as enzymatic subunits devoid of a cell binding domain, namely ribosomal inactivating proteins (RIPs) and include pokeweed anti-viral

protein, gelonin, modecin, and an inhibitor from barley or wheat germ and the like. Since protein synthesis is an essential cellular function, these toxins should have the ability to kill any cell to which the toxins are targeted, including cells normally resistant to chemotherapy or radiation treatment. The degree of selectivity in target cell killing of an immunotoxin is a function of the type of toxin, whether the whole toxin or only the portion of the toxin responsible for enzymatic activity is conjugated to the targeting moiety, the bond(s) used to conjugate the toxin to the targeting moiety, and factors affecting in vivo delivery. See Morgan et al., Antibody Immunoconjugates and Radio Pharmaceuticals, 2:165, 1989; and Morgan et al., Mol. Immunol., 27:273, 1990.

Toxins employed in immunotoxins as described above exhibit high toxicity, at least in holotoxin form, and high immunogenicity. Specifically, toxins adversely affect normal cells through receptor-mediated or non-specific uptake and, as a result, efficacy of the toxin against target cells is limited. Because these toxins inhibit an essential cellular function, e.g., protein synthesis, their non-target tissue uptake results in normal organ toxicity.

Administration of toxin also generates a strong immune response in vivo. As a result, the recipient's immune system recognizes the immunotoxin and removes it from the systemic circulation, thereby reducing the immunotoxin therapeutic efficacy. These problems represent barriers to the effective use of toxins for tumor treatment or for other therapeutic purposes.

As stated, immunotoxins are expected to exhibit no selectivity for metabolic processes within tumor

cells compared to normal tissues. Target cell metabolism may also be manipulated to selectively achieve a therapeutic goal, however. An example of such selectivity is evidenced by the non-targeted agents affecting cAMP dependent protein kinase and cAMP levels.

An example of a moiety capable of affecting cAMP dependent protein kinase (PKA) is 8-Cl-cAMP. The agent binds irreversibly to PKA, thereby halting proliferation of target cells which is dependent upon the enzyme. This agent has been demonstrated to inhibit proliferation of a variety of human and rodent tumor cell lines. Drug development aimed at identifying and/or designing drugs capable of discrimination between altered or amplified forms of protein kinase present in tumor tissues and in normal tissues is currently being pursued. In addition, anti-sense oligonucleotides to PKA have recently been shown to inhibit proliferation of tumor cells.

In addition to the direct acting agents, levels of cAMP and activation of PKA as well as other effector molecules within cells can be regulated indirectly through G-proteins. G-proteins are signaling moieties acting between certain receptors and effector molecules within cells. Ligand binding induces a signal which is communicated to effector molecules through modification and then subsequent interaction with the G-protein alpha subunits. The activated G-protein signals that the effector moiety should either commence or halt its effector function. A variety of different effector molecules, i.e., enzymatic or ion channel proteins, mediate changes in cellular processes. Generally, the receptors, G-proteins, and effector molecules are located in proximity to each other within the cell plasma membrane (Fig. 1). A portion of the receptor

typically extends beyond the cell surface to permit ligand binding. G-proteins are typically present as three-subunits, alpha, beta, and gamma. Effector molecules are generally capable of freely diffusing within the plasma membrane or cytoplasm and couple with an activated G-alpha subunit.

When a ligand binds to its receptor as shown in Fig. 1, the appropriate G-alpha subunit is activated by the exchange of GTP for GDP. The activated G-alpha subunit, which is released from association with the beta and gamma subunits, then interacts with an effector moiety, such as adenyl cyclase, to activate or deactivate the effector moiety. Activation of the effector adenyl cyclase, for example, results in the cleavage of ATP to form cAMP. When the receptor becomes unoccupied, i.e., the ligand-receptor interaction is lost, the G-alpha subunit will be de-activated and reassociate with the other G-protein subunits. Consequently, a loss of signal transduction will be experienced, and cAMP levels will return to normal.

In normal circumstances, the ligand will be bound to the receptor for only a short time. Consequently, the biological response induced by the receptor-ligand interaction is typically of short duration and is dictated by the concentration of ligand in proximity to a receptor.

A large variety of cellular responses have been shown to be mediated through G-proteins or changes in cyclic nucleotide levels by the use of specific bacterial toxins, namely pertussis and cholera. These toxins interact with different G-alpha proteins to uncouple receptor signaling of cAMP production (inhibitory) or to prolong the activated state of the G-alpha s subunit (stimulatory), thereby increasing cellular levels of cAMP. Thus, in this sense,

pertussis and cholera toxin may be viewed as the "yin and yang" for G-proteins. Using those toxins as analytical tools, investigators have examined specific target cells and receptor-ligand interactions to ascertain whether a given biological response is mediated through G-proteins.

The design of such studies is shown schematically in Fig. 2a. Target cells "T" are either pre-treated with toxin or simultaneously treated with ligand L and holotoxin "A/B." Holotoxin A/B binds through its B subunit to cellular acceptors, internalizes and catalyzes ADP-ribosylation of G-proteins thereby affecting signaling. A diminution or enhancement of the biological response triggered by ligand L binding to receptor "R" is taken as evidence of receptor-effector coupling through G-proteins. The results of studies carried out in this manner are given in Tables III and IV.

Effector moieties coupled to receptors through G-proteins are regulatable in accordance with the present invention. Exemplary effector moieties linked to receptors through G-protein substrates are adenyl cyclase, phospholipase, phosphodiesterase, ion channels, cAMP dependent protein kinase, and the like. The functioning of these exemplary effector moieties may be impacted by the toxin portion of conjugates of the present invention, operating through existing target cell signaling mechanisms.

Table III lists exemplary immunologic responses induced by ligand-receptor interactions and regulated by cyclic nucleotides or G-proteins.

TABLE III

EFFECTOR CELLS	RESPONSE	LIGAND
PMNs	Oxidative burst phagocytosis	FMLP
Platelets	Aggregation	Platelet-derived growth factor
T-Cells	IL-2 secretion	IL-1
	CTL activation and killing	Target cell
Monocytes	M-CSF synthesis	TNF
	IL-1 production	Pertussis toxin
Bone marrow Stem Cells (myeloid)	Proliferation	G-CSF, GM-CSF, IL-3, M-CSF, PGE
Hepatocytes, Monocytes, Fibroblasts, Endothelium	C1-inhibitor	Histamine, PGE-2, C5a des arg, Carbamyl choline, Phenylephrine, Interferons

Table IV lists exemplary non-immunologic responses induced by ligand-receptor interactions and regulated by cyclic nucleotides or G-proteins.

TABLE IV

EFFECTOR CELLS	RESPONSE	LIGAND
Glomerular epithelium	Growth	Leukotriene D4
Gastric parietal	Acid secretion	Histamine
Pancreatic islet	Somatostatin synthesis	Multiple polypeptide hormones
Adipocytes	Glucose Transport	Insulin
	Lipolysis	Pertussis Toxin

Ovary follicular	Ovulation	LH
Ovary cyto-trophoblasts	Progesterone secretion	Multiple polypeptide hormones
Sperm	Motility	Unknown
Multiple[?]	Phospholipase A2 Activity	EGF
Peripheral nerve	Growth	Forskolin
Adrenal glomerulosa	Steroid synthesis	Angiotensin
	Aldosterone production	ACTH
	ACTH Induced cAMP Production	IGF-1
Endothelium smooth muscle	Dilation (Relaxation)	PGE-E1
Endothelium	Prostaglandin synthesis	Alpha thrombins Histamine Arachidonate
Leydig cells	Steriodogensis	LH[,?] Angiotensin
	Inhibition of HCG synthesis	Corticotropin releasing factor (CRF)
Hepatocytes	Gluconeogenesis/ Glycolysis	Glucagon, Insulin, Catecholamines
Bone, Kidney tubule	CA^{++} mobilization	Parathyroid hormone (PTH)
Skeletal tissues	Growth	PTH
Cardiac myocytes (Pacemaker)	K^{+} channels	Muscarinic agonists
Cardiac Smooth Muscle	Ca^{++} mobilization Vasodilation Ventricular relaxation Contraction	Inhibitors of phosphodiesterase

Smooth Muscle (Multiple Tissues)	Contraction	ACTH
	Ca^{++} mobilization	Alpha-adrenergic and Beta-adrenergic agonists
	Ca^{++} dependent ATPase	Calmodulin

Pharmaceutical agents that can modulate the variety of biological responses listed in these tables may find significant utility. If such agents include an appropriate holotoxin, they would have the inherent advantage of high potency due to the enzymatic nature of the toxin. However, if advantage is to be taken of the ability of a holotoxin to diminish or amplify an in vivo biological response triggered by ligand binding, several obstacles must be overcome. First, the holotoxin is toxic to certain normal cells, limiting the toxin dose and therefore the amount of signal regulation achievable in target cells bound by the ligand. In addition, the holotoxin is not selective for receptor-positive target cells. Other cells to which the holotoxin might bind would compete with the receptor-positive cells for the toxin and limit its bioavailability and potency. Also, this method requires dual "receptor-ligand" interactions on the same target cell at the same time to function properly. Specifically, both ligand and holotoxin binding to the target cell must occur for the signal initiated by ligand binding to be amplified or reduced by the holotoxin.

Tumor cells exhibit aberrant and uncontrolled growth. One method of curing a mammal of a tumor is to kill the tumor cells while preserving the viability of the host. This is generally the approach of radiation therapy, chemotherapy and immunotherapy. Another approach is to cause the tumor cells to differentiate, since the

differentiated form of a cell is an end-stage cell. Although not generally successful, there have been anecdotes of cis-retinoic acid causing differentiation of human leukemia. In human male germ cell malignancies, chemotherapy may partially kill and partially cause differentiation into benign tumors. In tissue culture, nontoxic levels of cAMP in the media down-regulates certain cellular functions.

Summary of the Invention

As shown schematically in Fig. 2b, the present invention provides conjugates that retarget non-cytolytic toxin moieties to amplify or suppress differentiation functions of target cells. Conjugates of the first aspect of the present invention include an enzymatic portion of a non-cytolytic toxin capable of ADP-ribosylation of a G-protein and a targeting agent (ligand) capable of binding to a target cell receptor, which is coupled through G-proteins to effector molecules capable of impacting the desired biological response. Preferably, the receptor is at least somewhat specific to the target cells so that the ligand-BRM conjugate is at least preferentially delivered to those cells, such as tumor cells, for which manipulation is desired. Ligand, as a component of the conjugate, initiates intracellular signaling upon binding to the receptor. The signal is subsequently either amplified or uncoupled by action of the enzymatic portion of the conjugate, which is delivered to the target cell cytoplasm by internalization of the conjugate and released from the conjugate through a conditionally unstable linkage.

Toxin moieties and subunits derived therefrom useful in this aspect of the present invention

include toxins that do not affect protein synthesis directly but rather interact with G-proteins or modified G-proteins (oncogenes). Exemplary toxins from which enzymatic moieties can be derived are listed in Table V.

TABLE V

TOXIN	CELL BINDING SPECIFICITY	SUBSTRATE
Cholera	GM-1 ganglioside	Gs alpha
Pertussis	Glycoprotein	Gi alpha
E. coli heat labile enterotoxin	GM-1, GD1b, asialo-GM-1	Gs alpha
Botulinum Neurotoxins	multiple gangliosides	ras like proto-oncgene product

The conjugates of the present invention include toxin moieties having their endogenous cell binding capability nullified or removed by biochemical or recombinant genetic engineering methods or by occlusion occurring upon conjugation to the targeting agent. In this manner, the conjugate is retargeted in accordance with the cell binding specificity of the targeting moiety, rather than the endogenous cell binding activity of the toxin. In all instances, the final conjugate retains the enzymatic activity of the holotoxin.

Such retargeted conjugates are less toxic than the native holotoxin, because the conjugated toxin moiety no longer exhibits the endogenous cell binding specificity which elicits the normal dose-limiting toxicity pattern of the holotoxin. Moreover, targeting G-proteins and the cellular processes regulated by these proteins will not lead to cellular death in most cases but merely to an alteration of a particular biological response. Target cell

selectivity (i.e., bioavailability of the conjugate for target cells) is also improved by retargeting. Retargeting non-cytolytic toxins using a ligand specific for a receptor that is preferentially expressed on target cells will also impart a longer serum half life and higher bioavailability than holotoxin, as the receptors, for instance, for pertussis and cholera, are widely displayed over many cell types.

ADP-ribosylation of G-proteins by exemplary non-cytolytic toxins cholera and pertussis, is essentially irreversible and, as a result, the effects on the biological response are of extended duration with the effected cell recovering normal signal regulation only through synthesis of new G-proteins. Consequently, conjugates of the present invention employing portions of non-cytolytic toxins will be characterized by achieving a prolonged effect on the targeted biological response, with normal biological response recovery dictated by the rate of target cell G-protein synthesis.

A second aspect of the present invention provides conjugates capable of affecting functions of target cells independently of target cell signaling mechanisms and G-proteins. Such conjugates include a non-cytolytic toxin moiety capable of directly catalyzing an intracellular reaction that affects target cell function and a targeting moiety. Exemplary holotoxins useful in a direct-acting conjugate embodiment of the present invention are adenylate cyclase toxins. Such toxins catalyze the conversion of ATP to cAMP and are functionally equivalent to mammalian adenyl cyclase. Since these toxins do not operate through a cellular signaling system, the enzymatic portion of these direct-acting toxins may be retargeted by any moiety selective for

the target cells, without regard to whether the receptor for that moiety is linked to G-proteins. As a result, metabolic manipulation can be achieved in target cells, such as tumor cells, that may be abnormal in their signal transduction. These conjugates have the same features as those described above, being generally not directly toxic to non-targeted normal tissues as well as selective.

Some adenyl cyclase toxins, from which the toxin moieties employed in conjugates of this embodiment of the present invention are derived, are active for only a short time in target cells as a result of host cell enzymatic degradation of the toxin. Conjugates of this and other aspects of the present invention may therefore include toxin moieties modified to be less susceptible to degradation or inactivation, thereby maintaining elevated cAMP levels and altered biological responses for prolonged time periods.

A third aspect of the present invention is provided wherein a moiety derived from a non-cytolytic toxin capable of ADP-ribosylation of actin is conjugated to and retargeted by a targeting agent such as ligand or antibody. Such a conjugate can affect a variety of cellular processes, including proliferation through inhibition of actin polymerization and effects on the cellular cytoskeleton. These conjugates share the general properties of other conjugates of the present invention and may be similarly manipulated to improve conjugate performance.

Table VI lists exemplary toxins useful in the second and third aspects of the present invention.

TABLE VI

Toxin	Substrate
Adenyl cyclase toxins of Bordetella pertussis and Bacillus anthracis	ATP
Botulinum C2 toxins	actin
Clostridial iota toxins	actin

Conjugates of the present invention may also be manipulated by a number of methods to reduce the immunogenicity thereof, a problem inherent in the development of pharmaceutical products based on toxins.

Brief Description of the Drawings

Fig. 1 is a schematic representation of how G-proteins couple ligand-receptor binding to effector functions within cells.

Fig. 2a is a schematic representation of experiments performed to determine whether a given target cell receptor-ligand binding and a subsequent biologic response are coupled through G-proteins. The determination is made by pretreating or simultaneously treating a cell with a toxin that modifies G-protein activity in conjunction with ligand-receptor interaction.

Fig. 2b is a schematic representation of the retargeted, non-cytolytic toxin conjugates of the first aspect of the present invention.

Fig. 3 is a schematic representation of the structure of cholera holotoxin.

Fig. 4 is a schematic representation of a retargeted cholera toxin conjugate of the first aspect of the present invention.

Fig. 5 is a schematic representation of the structure of adenylate cyclase toxin (ACT) of Bordetella pertussis.

Fig. 6 is a schematic representation of recombinant derivatives of ACT useful in retargeted toxin conjugates of the second aspect of the present invention.

Fig. 7 is a schematic representation of a conditionally unstable peptide linkage useful in optimizing potency and selectivity of conjugates of the present invention.

Fig. 8 is a schematic representation of the cleavage of the peptide linkage of Fig. 5 within a target cell endosome.

Fig. 9 shows anti-toxin reactivity of normal human serum samples. Normal human donor sera were tested for reactivity with diphtheria toxin (DT), Pseudomonas exotoxin A (PE), pertussis holotoxin (PTx) and pertussis B oligomer (PTxB).

Fig. 10 schematically depicts antigen processing of a protein antigen (i.e., intact toxin) to small peptides that associate with MHC Class II antigens.

Fig. 11 shows the inhibition of proliferation in a mixed lymphocyte reaction (MLR) by cholera holotoxin (CTx) and cholera B oligomer (CTxB) (measured by ^{3}H-thymidine incorporation).

Fig. 12 shows a controlled charge modification reaction scheme which protects lysine groups of toxins that are important in biological function, while reducing the immunogenicity of conjugates of the present invention.

Description of Preferred Embodiments

Prior to setting forth the present invention, the definitions of certain terms used in the specification are provided.

Biological Response Modifier (BRM); Also Cytokine and Lymphokine: A moiety, e.g., a protein, that is capable of eliciting a functional response from target cells measurable in an in vitro or in vivo bioassay. A biological response might include alterations in differentiation functions, activation, induction or suppression of proliferation, inhibition or enhancement of secretion or de novo synthesis or alterations in synthesis of proteins, and the like. A BRM may enhance or suppress one or more of these functional responses.

Target Cells: A defined population of cells from which a biological response is to be elicited.

Retargeted Toxin: A toxin wherein the endogenous cell binding portion has been replaced by a different targeting moiety exhibiting preferential binding to target cells. The "replacement" is accomplished by biochemical or recombinant means and includes deleting, deactivating, blocking or otherwise nullifying the native BRM cell binding portion and conjugating the resultant toxin moiety by biochemical or recombinant means to a new targeting agent.

Non-Cytolytic Toxins: For the purposes of this specification, the term "non-cytolytic toxin" describes a protein toxin whose predominant affect is not to directly inhibit protein synthesis. Non-cytolytic toxins affect non-essential or differentiation functions carried out by target cells. Since non-cytolytic toxins do not affect protein synthesis, such moieties do not directly cause cell death in most circumstances. An exemplary

exception to this generalization is the process of apocytosis. The death of some T-cells, for example, is metabolically pre-programmed, and the program can be initiated by increased cAMP levels. Consequently, it might be expected that the generally non-cytolytic toxins that affect cAMP levels in target cells may cause the death of T-cells when targeted thereto. Also, some non-cytolytic toxins or conjugates thereof may halt proliferation of normally proliferating cells, such as tumor cells. Some of these non-proliferating cells may then die as a result of their inability to proliferate. Reduction in tumor size may result from inhibition of proliferation since tumor cell growth is a product of two processes, cell proliferation and death. A listing of exemplary non-cytolytic toxins, their substrates, and mechanism of action appears in Tables V and VI.

Cytolytic Toxin: A protein toxin moiety that inhibits protein synthesis, and is therefore directly capable of killing targeted as well as non-targeted cells demonstrating non-specific uptake. Conjugates derived from these toxins will demonstrate dose-limiting toxicities as a consequence of antigen-specific or non-specific uptake. Exemplary sources of such toxin moieties include plants (ricin, abrin, pokeweed antiviral protein, gelonin, and modecin), bacteria (diphtheria, Pseudomonas exotoxin A, and shigella), and marine organisms.

Targeting Agent: A protein, peptide, or non-proteinaceous organic molecule that preferentially binds to a population of cells intended for pharmaceutical manipulation (i.e., target cells). A targeting moiety may, for example, be a growth factor (e.g., and epidermal growth factor, a transforming growth factor, such as alpha or beta), a hormone, a cytocline, an antibody, a colony stimulating factor,

transferrin, bombesin, gastrin releasing peptide or the like. The targeting moiety may bind to a protein, glycoprotein, or glycolipid located on the target cell surface or an internal antigen. When the targeting moiety binds to a receptor and induces target cell signaling that leads to an alteration in target cell function, the targeting moiety is referred to as a ligand. Exemplary target cells are antibodies, fragments or functional equivalents thereof, such as those binding to the NR-LU-10 (an IgG_{2b} murine monoclonal antibody directed to the 37-40 kilodalton pancarcinoma glycoprotein), NR-ML-05 (IgG_{2b},k immunoglobulin may be generated in accordance with Woodhouse et al., "In Human Melanoma, From Basic Research to Clinical Application," Ferrine, ed., Springer-Verlag, pp. 151-163, 1990), and NR-CE-01 antigens (αCEA), and growth factors such as EGF, TGFα, TGFß, FGF, transferrin, interleukins 1-11, GM-CSF and G-CSF.

Preferred targeting agents useful in this regard are proteins, including antibody and antibody fragments; peptides, such as bombesin, gastrin-releasing peptide, cell adhesion peptides, substance P, neuromedin-B, neuromedin-C, and metenkephalin; and hormones, such as EGF, α-, and ß-TGF, estradiol, neurotensin, melanocyte stimulating hormone, follicle stimulating hormone, luteinizing hormone, and human growth hormone. Biotin, avidin, proteins corresponding to known cell surface receptors (including low density lipoproteins, transferrin and insulin), fibrinolytic enzymes, and biological response modifiers (including interleukin, interferon, erythropoietin and colony-stimulating factor) are also preferred targeting agents. Analogs of the above-listed targeting agents that retain the capacity to bind to a defined target cell population

may also be used within the claimed invention. In addition, synthetic proteinaceous targeting agents may be designed.

Function Equivalence: A first BRM, for example, is functionally equivalent to a second BRM if the BRM function of the first is achieved by the second. Consequently, non-cytolytic toxin conjugates capable of inducing a functional response from a target cell population are functionally equivalent, regardless of origin. A non-cytolytic toxin BRM purified from a natural source and conjugated to a targeting moiety is functionally equivalent to such a BRM conjugate formed from a fusion protein. Likewise, a holotoxin having an inactivated or occluded cell binding domain retargeted by a targeting moiety is functionally equivalent to a non-cytolytic toxin portion devoid of endogenous cell binding specificity. Similarly, a holotoxin, domain-sized or multiple domain-sized toxin portions, or a small peptide derived from or corresponding to a toxin portion of less than domain size that is purified from a native toxin or prepared by recombinant techniques may also be functionally equivalent when used in conjugates of the present invention.

Substrate: The moiety within target cells acted on by the non-cytolytic enzymatic toxin portion of the present invention.

ATP: Adenosine tri-phosphate.

ADP: Adenosine di-phosphate.

cAMP: Cyclic adenosine mono-phosphate.

GDP: Guanosine di-phosphate.

GTP: Guanosine tri-phosphate.

While the following description is set forth primarily in terms of chemical conjugates, the present invention also provides genetically

engineered fusion proteins that are essentially functionally equivalent to such conjugates. Consequently, a non-cytolytic toxin moiety includes conjugates, fusion proteins and the like.

As stated above, preferred non-cytolytic toxin portions useful in the present invention are derived from enzymatically active subunits or domains of the aforementioned protein toxins which do not affect protein synthesis directly. Such toxin portions may be of bacterial, marine, or plant origin or may be manufactured by recombinant techniques. Bacterial toxin moieties of the present invention are or may be derived from, for example, enterotoxins or exotoxins and may be of one or a multiple chain structure. A complete or whole toxin with both enzymatic and cell binding activity is referred to as a holotoxin. Toxin moieties need only exhibit a non-cytolytic function that results in alteration of a BRM activity or differentiation or slowing of cellular proliferation when appropriately targeted to a population of target cells to be useful in the present invention.

Cytolytic, protein synthesis-inhibiting toxins such as ricin, diphtheria, or Pseudomonas exotoxin A exhibit commonalities in function, but that functional commonality is not necessarily reflected by identical mechanisms of action or structural or sequence similarity. Such toxins include an enzymatic or catalytic portion, a translocating portion, and a cell binding portion. The enzymatic portion constitutes the toxicity generating portion of the toxin, i.e., catalyzes a modification of regulatory proteins or processes within a cell leading to inhibition of protein synthesis and eventually to cell death. Selectivity of cell killing has been previously achieved by removing the

cell binding portion and conjugation of the enzymatic portion to a different targeting molecule, namely an antibody or ligand having specificity for the target cell. The translocating portion of the cytolytic toxin is responsible for the internalization of that toxin into the target cell and transfer of the enzymatic subunit to the cytoplasm. The cell binding portion of the cytolytic toxin dictates the endogenous cell binding specificity of that toxin. The latter two functions may be present within the same polypeptide or subunit.

Similarly, non-cytolytic toxins express these three functional activities associated with protein domains. The enzymatic domain or subunit must be included in conjugates of the present invention to impart BRM activity thereto. When conjugated to a ligand suitable for target cell metabolic signaling, an appropriate enzymatic domain may, for example, either amplify or block the signal by interacting with G-proteins, thereby either enhancing or halting the biological response triggered by the ligand. Similar enzymatically active portions can be derived from non-cytolytic toxins that impact the metabolic processes of target cells directly without interacting through G-proteins. Such directly acting toxin moieties (e.g., the reaction catalyzed by the toxin moiety directly impacts target cell function) are the subject of the second and third aspects of the present invention.

ADP ribosylation reactions are central to the metabolic signaling mechanisms manipulated by retargeted conjugates of some aspects of the present invention. ADP ribosylation of certain substrates involved in the operation of intracellular signaling mechanisms results in the amplification or inhibition of target cell functional processes. Exemplary

substrates for ADP-ribosylation by bacterial or plant toxins are G-proteins, elongation factor-2 (EF-2), actin, rho-like oncogene proteins, and the like. Only toxins that act on EF-2 have been previously employed as portions of conjugates, however.

Exemplary toxin-substrate relationships that may be exploited by the present invention are shown in Tables V and VI are discussed below. Cholera toxin and E. coli heat labile enterotoxin cause ADP ribosylation of the G-protein alpha s. The endogenous cell binding specificity of cholera toxin is to GM-1 ganglioside, a receptor found on a plethora of cell types. E. coli heat labile enterotoxin exhibits endogenous cell binding specificity for GM-1, GD1b, and asialo-GM-1. Pertussis toxin induces ADP ribosylation of G-protein alpha i and exhibits endogenous cell binding specificity for glycoproteins that are ubiquitous on mammalian cells.

Botulinum neurotoxins act on ras-like proto-oncogene substrates. Ras oncogenes are similar to G-alpha proteins in function and may regulate tumor cell proliferation. A current theory endeavoring to explain this similarity is that such oncogenes are mutated G-proteins. Specifically, ras oncogenes are thought to be irreversibly activated G-proteins. Consequently, inhibition of ras function may lead to growth arrest of tumor cells. Botulinum neurotoxins exhibit endogenous cell binding specificity for many gangliosides. Conjugates or fusion proteins of these toxins affecting G-proteins or modified G-proteins and targeting moieties constitute an aspect of the present invention.

Specifically, this aspect of the present invention is directed to conjugates including a non-cytolytic toxin moiety and a ligand coupled to an

effector through G-proteins and capable of triggering a desired biological response. Non-cytolytic toxin moieties useful in conjugates of the first aspect of the present invention are toxin moieties that can alter a cellular function by modification of G-proteins and thus the existing target cell signaling mechanism. Exemplary non-cytolytic toxin moieties are portions of toxins, such as cholera toxin A1 subunit and pertussis toxin S1 subunit, in which the enzymatic function of the respective holotoxins reside. These exemplary non-cytolytic toxin moieties act on G-proteins, and thus on cellular signaling mechanisms which utilize receptors and effectors coupled through G-proteins. Functional processes subject to G-protein control therefore can be regulated by a non-cytolytic toxin portion of cholera or pertussis toxin.

The non-cytolytic portion of cholera toxin induces ADP-ribosylation of a G-alpha protein designated the stimulatory alpha protein (G-alpha s). Active G-alpha s (GTP form) may then interact with adenyl cyclase, inducing an increase in cAMP level. Normally, the activation signal is down regulated by enzymatic degradation of GTP to GDP, restoring the G-protein subunit to its inactive GDP form. Extended activation or suppression of G-protein mediated response may be caused by ADP ribosylation of the G-protein subunit by the non-cytolytic toxin. Increases in cAMP level may impact many target cell metabolic processes, such as inhibiting proliferation.

In contrast, the non-cytolytic portion of pertussis toxin uncouples the signaling mechanism through ADP ribosylation of a G-alpha protein designated either G-alpha i or G-alpha o. Specifically, effector moiety function is no longer

resultant from the ligand-receptor interaction. Under these conditions, ligand-receptor binding does not result in enhanced adenyl cyclase activity. Consequently, intracellular cAMP levels are not increased.

As a result, pertussis and cholera toxins are capable of suppressing or amplifying target cell metabolic or functional processes. See Tables III and IV for examples of these phenomena.

Both exemplary biochemical and recombinant technique-generated cholera toxin conjugates of the present invention are shown schematically in Figs. 3 and 4. The structure of cholera toxin is shown schematically in Fig. 3. The toxin subunit exhibiting enzymatic activity is designated "A1," and is linked through a disulfide bond to a smaller peptide "A2." For producing a conjugate, the enzymatic toxin portion, A1, is joined to a targeting moiety "TP" by a disulfide bond which is cleavable by reduction in endosomes.

Non-cytolytic toxins that do not act on G-proteins are also useful in the present invention. Such toxins are capable of directly modulating cyclic nucleotide levels (second aspect of the present invention) or modifying cellular actin (third aspect of the present invention).

Conjugates or fusion proteins of the second aspect of the present invention involve, for example, adenylate cyclase toxins of Bordetella pertussis and Bacillus anthraces that have enzymatic activity functionally similar to mammalian adenyl cyclase. Insertion of this toxin protein within target cells will thus act directly on ATP to increase intracellular cAMP levels. Such toxins can thereby affect functional activities regulated by cAMP. The toxicity of such bacterial enzymes is directed to

peripheral leukocytes, namely PMNs, but retargeting will reduce this otherwise dose-limiting toxicity.

The structure of the adenylate cyclase toxin of Bordetella pertussis is schematically shown in Fig. 5. Like other toxins, adenylate cyclase toxins exhibit catalyzing, translocating, and cell binding functions. The catalytic (i.e., enzymatic) function requires mammalian calmodulin for activity and is associated with a domain located between amino acids 1 and 450. The translocation function is likely provided by a toxin domain located between amino acids 450 and 1000. The cell binding function is likely provided by a toxin domain located between amino acids 1000 and 1600. In addition, the toxin has protease-sensitive sites in the structural region between amino acid 450 and amino acid 1600. As a result, when administered as a whole toxin, the toxin is rapidly inactivated by target cell proteases.

Exemplary recombinant derivatives of the adenylate cyclase toxin of Bordetella pertussis are schematically shown in Fig. 6. The longer derivative corresponds to the enzymatic and translocating domains of the adenylate cyclase toxin, while the shorter derivative includes only the enzymatic domain. The impact of target cell protease activity on the conjugates is reduced by use of the shorter derivative, thereby prolonging the effective duration of conjugate activity. However, the potency of a conjugate containing a toxin portion devoid of the translocating domain may be less than that of a holotoxin. Thus, testing of both forms of the conjugate is necessary to determine the form with optimal potency. In addition, the relative value of each described moiety will vary according to the targeting molecule; for example, a rapidly internalizing targeting molecule complex will be more

active than one that internalizes slowly when using the molecule devoid of the translocating domain. Alternatively, the smaller derivative can be utilized with an artificial translocating peptide specified by Copending U.S. Patent Application No. 07/232,337, entitled "Covalently-Linked Complexes and Methods for Enhanced Cytotoxicity and Imaging, filed August 15, 1988. Conjugation of the adenyl cyclase toxin portion and targeting moiety is preferably achieved through the peptide linkers described herein, or as fusion proteins.

In addition, protease sensitive site(s) in native or recombinant Bordetella pertussis toxin or portions thereof could be identified and modified by site-specific mutagenesis, so that those sites would no longer be cleaved by target cell proteases. Alternatively, the protease-sensitive sites may be deleted.

B. anthraces toxin appears to internalize within cells through a receptor-mediated event. As a result, the B. anthraces toxin may be more useful for conjugation, as the enzymatic subunit may be more easily separated from cell binding activity.

Actin polymerization is important to a number of cellular processes including secretion (i.e., exocytosis) and proliferation. ADP ribosylation of actin has been shown to inhibit actin polymerization. Botulinum C2 toxins and Clostridial iota toxins act on (i.e., induce ADP ribosylation of actin), thereby more directly affecting target cell function (i.e., effect achieved with no G-protein or modified G-protein intermediary). The endogenous cell binding specificity of these toxins is unknown. The third aspect of the present invention features conjugates or fusion proteins of non-cytolytic toxins capable of ADP ribosylation of actin and targeting moieties.

Unlike conjugates of the first aspect of the present invention, the BRM activity of conjugates of the second and third aspects need not be targeted with ligands capable of triggering responses mediated through G-proteins. As a result, a broader array of ligands or targeting proteins can be utilized to target the cell population in which the conjugates will effect a biological response upon internalization. The biological response(s) elicited will be a function of the target cells and the differentiation functions that those cells carry out.

In some circumstances, the inclusion of the translocating domain of a non-cytolytic toxin in conjugates of the present invention may be necessary to allow full conjugate potency. In these cases, the rate and degree of target cell internalization and egress from target cell endosomes into the cytoplasm could be enhanced by the toxin translocating domain.

For example, holotoxins (i.e., whole toxins) may be used in constructing conjugates of the present invention. Conjugation of holotoxins to targeting moieties must be achieved in a manner such that the endogenous cell binding portion of the holotoxin is at least partially occluded in some manner, thereby preventing the endogenous toxin cell binding portion from interacting with endogenous target cells. Methods appropriate for achieving the retargeting of holotoxins are discussed in U.S. Patent Application Serial No. 559,863, entitled Immunological Effector Molecules Derived From Toxins," filed July 27, 1990.

In other situations, inclusion of the translocating domain within the conjugate may not garner an appreciable advantage, because the targeting portion of the conjugate may be sufficient, through binding with its receptor, to cause internalization of the conjugated enzymatic portion of the non-

cytolytic toxin. Moreover, inclusion of the translocating domain may increase the immunogenicity of the conjugates. This problem may be mitigated by the techniques for decreasing immunogenicity set forth below. A practitioner in the art would therefore be able to determine the portion of the toxin structure to be included in a conjugate of the present invention useful to achieve an optimal specific metabolic response in a specific set of target cells.

Also important to the potency of a conjugate of the present invention is the ability to release the toxin portion from endosomal vesicles into the target cell cytoplasm, allowing it to interact with its substrate. Such a releasing mechanism may be already present in the structure of a holotoxin. In some cases, the sequence active in release is found near the enzymatic subunit or domain. For example, diphtheria toxin likely contains such a sequence, since a portion roughly corresponding to the enzymatic domain is released into the cytoplasm while the B chain is required for optimal conjugate potency (binding and translocation). Similar findings have been made with respect to Pseudomonas exotoxin A and cholera toxin. In the latter case, a proteolytically sensitive state may exist in the A2 peptide adjacent to the enzymatic A1 subunit.

In order to ensure optimal potency of conjugates containing such toxin moieties, recombinant deletion variants of the B-chain in combination with the enzymatic domain are constructed and tested for potency. Alternatively, fusion proteins or chemical conjugates of such deletion variants may be constructed and similarly tested. In this manner, the smallest portion of the B-chain able to confer optimal potency is identified. Since

optimal potency requires the release mechanism, one also simultaneously identifies the portion(s) of the B chain with the sensitive sequence.

In both conjugates and fusion proteins of the present invention, potency may also be reduced as a result of steric hindrance of the targeting moiety in its interaction with its receptor. Consequently, a linker is preferably provided to separate the targeting moiety and toxin portion and allow the targeting moiety to bind to its receptor.

Exemplary protein to protein conjugation methods are disclosed in co-pending U.S. Patent Application Serial No. 095,178 (allowed but not yet issued) and U.S. Patent Nos. 4,367,309, 4,468,382, 4,363,758, 4,450,154, and 4,698,420. Commercially available linkers are described, for example, on pages E-2 through E-19 of the Pierce 1990 ImmunoTechnology Catalog and Handbook.

Conjugates of the present invention may therefore be linked by disulfide and thioether linkages will as well as by peptide bonds. An exemplary linking procedure involves two steps: (1) reacting the toxin portion and the targeting moiety with the same or different hetero or homobifunctional cross-linker to form a derivatized toxin portion and a derivatized targeting moiety, and (2) reacting the derivatized toxin portion and the derivatized targeting moiety to obtain a covalently-linked conjugate. Conjugate species are then purified from excess chemical linker or unreacted toxin and targeting moiety by one or more of a variety of chromatographic procedures. A practitioner in the art could design and implement appropriate conjugation and separation procedures.

Specifically, native sulfhydryl groups located on the toxin portion or the targeting moiety may be

reduced, for example, with DTT (dithiothreitol) to form derivatized moieties for use in step (2) described above. Also, a sulfhydryl moiety may be introduced into the targeting moiety and a maleimide moiety may be introduced into the toxin portion through derivatization of lysine groups with a heterobiofunctional reagent. A condensation reaction of such derivatized components may then be undertaken to form conjugates of the present invention. See, for example, U.S. Patent No. 4,981,979.

For example, the following commercially available agents may be used to derivatize the toxin BRMs and/or targeting moieties of conjugates of the present invention:

Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC).

Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC).

m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS).

m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Sulfo-MBS).

N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP).

Succinimidyl 4-(p-maleimidophenyl) butyrate (SMPD).

Sulfosuccinimidyl 4-(p-maleimidophenyl) butyrate (Sulfo-SMPD).

N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB).

Sulfosuccinimidyl (4-iodoacetyl) aminobenzoate (Sulfo-SIAB).

Recombinant procedures may be employed to provide fusion proteins of non-cytolytic toxin moieties and a proteinaceous ligand or targeting agent. Exemplary fusion protein production methods

are illustrated in Bailon et al., Biotechnology, 6:1326, 1988; Chaudhary et al., Proc. Nat'l Acad. Sci. (USA) 84:4538, 1987; Siena et al., Blood, 72:756, 1988; Chaudhary et al., Proc. Nat'l Acad. Sci. (USA), 87:1066, 1990; Chaudhary et al., Nature, 339:394, 1989; Pastan and Fitzgerald, J. Biol. Chem., 264:15157, 1989; Berger et al., Proc. Nat'l Acad. Sci. (USA), 86:9539, 1989; and Williams et al., Protein Eng., 1:493, 1987.

Disulfide linkages may be prematurely cleaved in vivo prior to delivery of the conjugate to target cells, however. A preferred peptide linker useful for constructing fusion proteins in accordance with the present invention is shown in Fig. 7. A toxin enzymatic portion, encoded by a nucleotide sequence within a fusion protein and expressed in an appropriate prokaryotic or eukaryotic system and designated "A," is bound to a targeting moiety designated "T.P." by a sequence encoding a peptide linker. The peptide linker includes residues capable of specifying flanking spacer regions, terminal glycine residues, and a disulfide bond loop containing a consensus sequence sensitive to endosomal or lysomal enzymes. The peptide linker both separates the enzymatic subunit from the targeting moiety and provides for enzymatic subunit release upon target cell internalization.

Amino acid residues sensitive to lysosomal and endosomal protease cleavage are known. An example of such a sequence is given by the single amino acid code KWVEE. The indicated sequence can be cleaved by a variety of endosomal or lysosomal enzymes including cathepsins A through H. The availability of the sequence to enzymes located within target cell endosomes is enhanced by the presence of that sequence within a disulfide bonded loop. Moreover,

the disulfide bond is protected from premature reduction by liver disulfide isomerase or by serum glutathione by the peptide sequence which is insensitive to such serum enzymes. Alternatively, the entire cholera toxin A2 sequence may be encoded into the fusion protein instead of the simpler peptide sequence. Based on previous data, this is the site likely to be the endogenous site of cleavage within cholera toxin.

The release of the biological response modifying toxin portion A within target cell endosomes is shown schematically in Fig. 8. When a conjugate or fusion protein of the present invention is internalized within a target cell endosome, the conjugate is subjected to low pH, a reducing environment and endosomal proteases such as cathepsins. Endosome proteases cleave the sensitive peptide linker. The disulfide bond portion of the dual linker then undergoes reduction allowing release of the enzymatic domain of the conjugates of the present invention from the targeting moiety.

As indicated previously, non-cytolytic toxins, such as those capable of interacting with target cell metabolic signaling mechanisms, exhibit cell binding specificity for a variety of target cells. To increase the bioavailability of toxin enzymatic activity for receptor positive target cells, the portion of the toxin responsible for cell binding is removed and substituted with a targeting moiety having a receptor that is enriched on the target cell population. The increase in bioavailability may alternatively be achieved by inactivation or blockage of the endogenous cell binding specificity and toxin retargeting with a more application-appropriate targeting moiety.

The retargeted conjugates of the first aspect of the present invention only induce biological responses within receptor positive cells. In addition, these conjugates will only affect differentiation rather than essential cellular functions within target cells. Consequently, with a few exceptions, the conjugates of the first aspect of the present invention are non-toxic to target cells. Non-receptor bearing cells that are non-specifically targeted by conjugates of the present invention, would not likely be affected at all, because a receptor-triggered event must occur in combination with an enzymatic modification of G-proteins to produce the biological response. Internalization by pino- or phagocytosis of conjugate would not induce a response, as signaling would not have been triggered by ligand binding.

An example of such selectivity of a conjugate is illustrated with cholera toxin. Cholera toxin binds to GM-1 ganglioside, a moiety present on many normal tissues. The toxicity of cholera toxin is, however, only manifested after binding to GM-1 ganglioside located on colonic epithelium. Such binding induces active water resorption and acute diarrhea. Removal or inactivation of the cholera toxin B chain eliminates endogenous binding and the adverse effects thereof. Substituting IL-2 for the B chain allows targeting of cholera toxin to IL-2 receptor positive T-cells. Since IL-2 receptors are not located on the colonic epithelial cells, no dose-limiting diarrhea will be exhibited by the conjugate.

A bacterial toxin, such as Pseudomonas exotoxin A (PE), may be derivatized to reduce immunogenicity. Fig. 9 shows that, although most individuals have not been exposed or vaccinated to PE, occasional "normal" humans have pre-existing

anti-PE antibodies. For both pertussis toxin and PE, pre-existing antibody is therefore generally of little concern. In contrast, pre-existing antibody would be of concern if one utilized diphtheria toxin in conjugates of the present invention.

To permit multiple administrations of a toxin BRM conjugate of the present invention, marked reduction of immunogenicity is essential to avoid invoking a high titer neutralizing antibody response by the recipient's immune system. Several approaches to reducing immunogenicity may be used either singly or in combination, and exemplary approaches are set forth in Table VII.

TABLE VII

1. Administer doses below the threshold for immunogenicity.
2. Reduce BRM complexity (i.e., remove extraneous portions of the toxin molecule not essential for BRM activity).
3. Map immunodominant sites and modify by recombinant deletion or site-specific alteration.
4. Administer the conjugates to immuno-suppressed (e.g., chemotherapy- or radiation-treated) patients.
5. Chemically modify the BRM to decrease immunogenicity.

Regarding the first approach, mouse models may be used to estimate the immunogenicity threshold for a toxin conjugate. For example, about 2 μg of pertussis toxin B oligomer plus adjuvant has been shown to be necessary to invoke an optimal immune response in a mouse model. Lower doses that are sufficient to induce a biological response will therefore be poorly immunogenic. Chronic

administration over a period of months, however, will likely induce an immune response.

Under the second approach, portions of a toxin molecule are deleted through recombinant methods so as to eliminate non-BRM active portions of the molecule.

The third approach features identification and modification of the specific epitope(s) of the toxin molecule predominantly responsible for the anti-toxin response. For instance, immunodominant sites of Pseudomonas exotoxin A and pertussis toxin have been associated with the enzymatic subunit or domain. Genetic manipulation may be used to alter (by deletion or site-specific mutagenesis, for example) these immunodominant sites within the enzymatic subunit, resulting in a toxin derivative possessing reduced immunogenicity. The resulting mutagenized toxin would still be immunogenic, but would likely not induce as rapid or as high-titered responses as native toxin using the same dose of immunogen.

In lieu of modifications to the toxin per se, the fourth approach contemplates administration of immunogenic unmodified toxin to a "modified recipient" population that is unable to generate a normal and vigorous immune response (for example, patients that are immunosuppressed due to disease such as AIDS, and/or treatment such as cancer chemotherapy).

The fifth approach involves chemical treatment that modifies certain amino acid residues of the toxin, thereby producing a derivative that elicits little or no anti-toxin response while retaining functional BRM activity. Exemplary methods in this regard are described in co-pending U.S. Patent Application Serial Nos. 07/157,213 and 07/330,846, hereby incorporated by reference in their entireties.

An additional approach involves derivatizing the toxin or fusion protein with polyethyleneglycol.

Cationization of proteins has an enhancing effect on antigen presentation and immunogenicity. Conversely, anionic modification of proteins may be employed to create a relatively homogeneous distribution of negative charges on the surface of the toxin. Such anionic modification may reduce the ability of macrophages to metabolize and present degradative peptides to immunocompetent cells. Similar to polymers having repetitive sequences, toxins having a homogeneous charge distribution may not be well-recognized by T-cell dependent mechanisms. These consequences of charge modification result in a relative decrease in toxin immunogenicity.

Charge modification is particularly advantageous in instances where immunodominant amino acids or sequences are difficult to identify or predict. Toxins undergo antigen processing when injected into a mammalian recipient. Antigen processing involves degradation of the intact toxin molecule to small peptides (Fig. 10). These small peptides associate with MHC Class II (HLA-DR) antigens of the recipient's immune system on the surface of recipient's antigen presenting cells. The small peptides must "fit" the HLA-DR structure (i.e., have the proper amino acid sequence to interact with appropriate amino acids within the HLA-DR antigen).

While hydrophobic and charged amino acids are often associated with immunodominant sequences of antigens, immunodominance may vary with the haplotype of the individual recipient, especially in outbred populations (i.e., humans). Consequently, identification of consensus immunodominant sequences may be difficult. Modification of specific amino acids or

sequences to reduce immunogenicity may therefore be so complex as to become infeasible.

An exemplary means for reducing toxin immunogenicity through chemical modification involves treatment of the toxin with succinic anhydride. Succinic anhydride can modify the following amino acid residues under appropriate conditions: lysine, histidine, tryptophan, serine, threonine, and cysteine (free SH form). Amino acid residues believed to be essential for biological/chemical activities of ADP-ribosylating toxins include lysine (cell binding and possibly translocation), glutamic acid (NAD binding), tyrosine (NAD binding) and histidine or tryptophan (substrate binding). In the case of PE, a histidine residue essential for substrate binding is inaccessible in the absence of toxin unfolding, so succinic anhydride exposure would not modify this essential residue. Glutamic acid is also not modified by succinic anhydride. The lysine residues of PE, however, are more problematic. A practitioner in the art, however, would be able to minimize lysine modification through alteration of pH and reactant levels. Alternatively, lysine protecting and deprotecting steps can be used in a succinylating methodology, such as that discussed in Example II below, to overcome this problem.

As discussed previously, efficacious anti-tumor agents have been developed which act to inhibit cAMP regulated cellular processes such as proliferation, differentiation, or the process of metastasis. Although such agents have shown utility, they may suffer from less than adequate potency and short pharmacologic half life.

Conjugates of two aspects of the present invention are useful for modulating the intracellular target cell cAMP level and provide for high potency

and relatively long serum half life compared to small molecules. When the target cells are tumor cells, elevated cAMP levels will result in inhibition of proliferation or induction of differentiation. Such anti-tumor effects are known to be elicited by agents that modulate cAMP levels in a stoichiometric fashion, including the alkylating inhibitor 8-Cl-cAMP, agonists or antagonists of phosphoinositol production, chemical inhibitors of cAMP dependent protein kinase (PKA) and antisense oligonucleotides for inhibition of PKA. As a class, these stoichiometric agents are relatively non-toxic.

Since tumor cells are abnormal, different therapeutic approaches than those envisioned for normal cells should be contemplated for treatment including conjugates of the second aspect of the present invention. Tumor cells often undergo de-differentiation upon neoplastic conversion. Normal signal transduction and/or receptors may be lost in this process. Exemplary of such a loss upon neoplastic conversion is the p21 H-ras oncogene. This oncogene bears a resemblance to a G-alpha protein; however, p21 H-ras oncogene exists permanently in the active state. To treat tumor cells with such altered signaling mechanisms, the tumor cells could be targeted with a conjugate utilizing targeting moieties such as epidermal growth factor (EGF), transforming growth factor, such as alpha or beta, transferrin bombesin, gastrin releasing peptide or monoclonal antibodies specific to tumor cells and an enzymatically active toxin moiety derived from the adenyl cyclase toxins of B. pertussis or B. anthraces. Increasing the level of intracellular cAMP can be achieved in this manner, thereby causing differentiation or halting proliferation of tumor cells. Conjugates of the

second aspect of the present invention achieve an elevation in intracellular cAMP level independently of G-protein mediated signaling processes and, thus, are not subject to alterations in signal transduction mechanisms resulting from neoplastic transformation.

Certain advantages are garnered by the use of conjugates capable of elevating cyclic nucleotide levels rather than conventional stoichiometric (i.e., chemical) regulators thereof. For example, such conjugates have a higher potency than agents which act stoichiometricly since they are enzymatic or catalytic in nature. Conjugates of the second aspect of the present invention, for example, are expected to induce a target cell metabolic response at a concentration of about 10^{-11} M (in vitro), based on the demonstrated potency of conjugates that utilize cytolytic toxins. In contrast, stoichiometric modulators of cAMP levels, such as forskolin, require a concentration of about 10^{-6} M for activity. Also, conjugates of the present invention, especially of the first aspect, generate a metabolic effect of prolonged duration (e.g., hours to days as opposed to minutes). In contrast stoichiometric modulators usually express activity for only as long as sufficient levels are maintained at the cellular or tissue level. In addition, conjugates of the present invention may be formed of a relevant toxin domain and a chemical or proteinaceous targeting moiety specific for a certain population of target cells. As a result, the conjugates of the present invention may be designed to induce a specific metabolic response in a specific target cell population, thereby allowing flexibility in therapeutic design and known mechanism of action. Stoichiometric modulators, on the other hand, have required extensive research to determine structure-function

relationships and mechanisms of action and are relatively non-specific.

Applications using the conjugates of the present invention in cancer therapy are dependent upon the presence of a toxin portion capable of causing tumor cell growth arrest and/or differentiation. Cancer therapy applications for the conjugates of the present invention are listed in Table VIII.

TABLE VIII

Tumor cytostasis/killing
Differentiation of tumor cells, especially clonigenic cells, into end-stage, non-clonigenic cells
Inhibition of metastases
Upregulation of tumor associated antigen receptors for improved antibody or ligand targeting
Enhanced drug accumulation and thus increased chemotherapy efficacy
Reversal of drug resistance
Enhanced humoral and cellular immune responses

An inhibition of tumor cell proliferation can result in cell death. In addition, an increase in tumor cAMP level has been shown to inhibit the ability of the cells to metastasize. Increased cAMP levels have also been shown to increase chemotherapeutic drug accumulation in tumor cells through the reversal of drug resistance. Patients with germ cell malignancies have been treated with cytotoxic chemotherapy (generally, cis-platinum, vinblastine and bleomycin). Although the intent of this treatment is to kill the cells, in some patients neoplastic tumors differentiate into benign tumors

that are then excised. These patients are often cured. Similarly, non-cytolytic, retargeted toxins of the present invention can induce differentiation in the tumor cells.

Tumor cells are poorly immunogenic. Tumor cells that differentiate, however, may induce or express higher levels of differentiation antigens. These differentiation antigens can be highly immunogenic. The cancer patient's humoral and cellular immune system could mount a greater response to the differentiated tumor cells than to the undifferentiated cells.

Differentiation of tumor cells can also be a strategy to enhance the efficiency of other therapies. For example, the estrogen receptor status of breast carcinoma has been shown to be an independent prognostic variable. More importantly, the level of expression of this receptor on tumor cells predicts the response of the tumor to treatment with tamoxifen, a synthetic anti-estrogen. Breast tumors with high estrogen receptor expression respond much more frequently to tamoxifen treatment than those with low or absent levels of the receptor. A retargeted toxin of the present invention may be utilized to cause differentiation of the tumor cells rendering them more estrogen-dependent, permitting the tumor cells to respond adequately to subsequent tamoxifen treatment.

Another example is the HER-2/neu oncoprotein whose expression is increased in poor prognosis breast cancer. An antibody/non-cytolytic toxin conjugate or fusion protein of the present invention can be used to target these cells and cause them to differentiate or retard their proliferation.

Additional antigens, such as TAA (Tumor Associated Antigens) or other receptors, may also be

upregulated as a result of a cAMP level increase in tumor target cells. Upregulation of TAA could improve anti-TAA antibody binding to the tumor cell. Consequently, the effectiveness of passive administration of diagnostic or therapeutic antibodies will be enhanced. For example, therapeutic or diagnostic conjugates employing antibody targeting agents will bind at higher levels to differentiated tumor cells, thereby enhancing the therapeutic or diagnostic effect. Upregulation of receptors can also enhance receptor-ligand binding. Subsequent therapies involving radioactive or otherwise conjugated ligand may be rendered more effective in this manner.

Alternatively, conjugates of the first aspect of the present invention, designed to increase the intracellular level of cAMP of tumor cells through modification of proteins and signaling mechanisms, may be efficacious against tumor cells that have maintained a normal signaling mechanism. The existence of such a normally functioning, metabolic signaling mechanism must, however, be established and shown to be maintained within multiple tumors of the same histological type and capable of eliciting differentiation or proliferation inhibition.

For example, conjugates or fusion proteins of cholera toxin A1 (enzymatic subunit) and EGF, TGF-alpha, transferrin, or antibody to TAA may be employed to induce differentiation or inhibition of proliferation of tumor target cells. Such conjugates need to be shown to induce differentiation or inhibition of proliferation <u>in vitro</u>. Effective conjugates are subsequently evaluated in animal models of neoplastic disease using a therapeutic protocol (<u>i.e.</u>, taxomifen therapy of breast cancer) to determine <u>in vivo</u> efficacy.

There are a variety of other applications for the retargeted non-cytolytic toxins of the present invention. For example, toxin BRM conjugates of the present invention having proliferative activity may be advantageously used, singly or in combination with other agents, for the treatment of AIDS or HIV infection.

Pertussis toxin has been known to induce a lymphocytosis. The enzymatic activity of the toxin is required for this purpose. Recent data indicates that while pertussis toxin is capable of stimulating the proliferation and subsequent maturation of both CD-4 and CD-8 positive T-cells, the latter predominate in long term culture. To overcome this propensity as well as the neurological toxicity associated with pertussis toxin, an IL-7 targeted pertussis S1 enzymatic subunit conjugate or fusion protein may be utilized. Retargeting eliminates the normal dose-limiting toxicity and provides a BRM known to act on early lymphoid progenitors. Enzymatic modification of G-proteins in such progenitors enhances the proliferative activity of IL-7 resulting in increased production in the bone marrow and, with maturation in the thymus, increased levels of CD-4 positive T-cells in the periphery.

If these conjugates or fusion proteins are administered in conjunction with an agent capable of inhibiting virus replication, such as azathiopyrimidine (AZT), BI-RG-587, CD-4 peptides or functional equivalents thereof, the combination therapy will lead to a recovery of virus negative CD-4 positive T-cells and elevated CD-4/CD-8 ratios. Alternatively, combination therapy involving a conjugate or fusion protein of the present invention and an HIV infection inhibiting agent, such as CD-4/Ig6, might be employed.

Another potential therapeutic application involves a CD5-targeted pertussis or cholera toxin conjugate. CD-5, though expressed on virtually all T-cells, is present also on a subpopulation of B-cells, which are continuously renewing and likely responsible for production of certain auto-immune antibodies and perhaps auto-immune diseases. Inhibiting proliferation of such cells or engendering a decrease in auto-immune immunoglobulin may thus lead to the resolving of auto-immune disease. In fact, early success has been reported in treating rheumatoid arthritis with a CD-5/ricin A chain conjugate. This conjugate, as is typical of conjugates utilizing portions of cytolytic toxins, demonstrates dose limiting hepatic toxicity. Thus, conjugates of CD-5 and pertussis S1 or cholera A1 could inhibit proliferation of CD-5 positive B-cells without giving rise to hepatic toxicity.

The conjugates of the present invention are intended for injection into humans or other mammalian recipients. Accordingly, appropriate manufacturing and in vitro storage practices must be observed to provide suitable sterile, pyrogen-free compositions. Although not necessary, it is preferable to use a pharmaceutically acceptable extender or filler to dilute any carrier which might optionally be used and/or to simplify metering the requisite small quantities of such compounds. Sodium chloride and glucose are preferred carriers; sodium chloride is especially preferred because it facilitates provision of an isotonic solution.

An effective dose of a conjugate of the present invention is an amount sufficient to elicit a therapeutically meaningful biological response in a recipient. An appropriate dose would range from about 1 milligram to about 10 grams and be

administered by intravenous, intraperitoneal, subcutaneous, intramuscular, or other routes of administration. Of course, the exact dosage and administration route will vary depending on the nature of the patient's ailment, conjugate components used, and the like. A practitioner in the medical arts would be able to determine an appropriate dose and administration route for conjugates of the present invention.

The following Examples are presented for illustrative purposes only and do not therefore limit the scope of the present invention in any way.

EXAMPLE I

Process for Identification of Toxins and Enzymatic Domains Thereof with Activity Suitable for Construction of Conjugates of This Invention.

A. Identification of a Toxin with appropriate BRM Activity.

Cholera toxin has been previously described to be suppressive for lectin-stimulated proliferation of peripheral blood mononuclear cells in vitro. In addition, recent studies have indicated that cholera toxin can enhance renal allograft survival.

The test results have been reported in Tsugita et al., Transplantation, 48:1064, 1989. Results of that testing indicate that whole cholera toxin suppressed kidney rejection leading to enhanced survival time when compared to cyclosporin (Sandoz). Moreover, cholera toxin was much more potent (about 700 fold) and easier to administer, i.e., a 100 ug/kg dose i.v., single administration as opposed to 14 daily administrations of 5 mg/kg i.m. of cyclosporin. These results indicate that a bacterial toxin characterized by suppression of T-cell

proliferative responses in vitro may exhibit a BRM activity suitable for in vivo control of transplant rejection.

B. Identification of the Domain of Cholera Toxin Responsible for BRM Activity.

Experimentation was also conducted in vitro using mixed lymphocyte reaction (MLR) and cholera toxin components. This in vitro procedure constitutes a corollary to in vivo renal allograft testing. The results of these experiments are shown in Fig. 11 and are discussed below.

Fig. 11 shows the performance of cholera holotoxin (CTx) and the B oligomer of cholera toxin (CTxB) with respect to the inhibition of MLR proliferation. As expected, when histoincompatible lymphocytes were admixed, proliferation occurred. That proliferation was not appreciably arrested by the addition of CTxB to the mixed lymphocyte culture. However, a marked reduction in proliferation was observed when CTx was added to the mixed lymphocyte culture. These results indicate that the domain responsible for inhibition of proliferation is associated with the toxin's enzymatic activity and likely the A1 subunit. To obtain a conjugate with the appropriate BRM activity, the enzymatic or A1 subunit is therefore required.

C. Construction of Cholera Toxin A1/IL-2 Conjugate for Abrogation of Transplant Rejection.

Cholera toxin has been shown to be both able to abrogate proliferation of T-cells and bind to colonic epithelium, causing acute diarrhea. Consequently, cholera toxin cannot be administered at a dose sufficient to achieve optimal immunosuppression of T-cells. Removal of the B-chain and retargeting the

toxin A-chain with IL-2, i.e., interleukin-2, would allow specific suppression of activated T-cells without dose-limiting diarrhea.

Cholera toxin A1 subunit (CTxA1) is purified and isolated in accordance with conventional procedures. See, for example, Dosio et al., Antibody Immuno-Conjugates and Radio-Pharmaceuticals, 2:101, 1989. CTxA1 (1 mg/ml) in 0.1M NaCl, 0.1 M sodium phosphate buffer (pH 7.5) is mixed with a ten-fold excess of N-succimidyl 3-(2-pyridyldithio)-propionate (SPDP) dissolved in a small amount of absolute alcohol for about 2 hours at room temperature. The mixture is passed over a PD-10 gel filtration column (Pharmacia) equilibrated in 0.15M NaCl, 0.1M sodium phosphate (pH 7.5) (PBS) to remove unreacted SPDP. IL-2 is similarly reacted with SPDP. Derivatized CTxA1 is then treated with 50 mM dithiothreitol (DTT) to generate a free SH moiety and then desalted. Derivatized and reduced CTxA1 is mixed with derivatized IL-2 and allowed to react at room temperature for a time period extending from about 6 hours to overnight. The conjugate is then chromatographed on a TSK-3000 column (Waters) using an FPLC system (Pharmacia) to separate conjugate from unreacted materials. Conjugate-containing fractions are then pooled and may then be used, for example, for in vivo allograft rejection studies or treatment.

EXAMPLE II

Modification of Retargeted Toxin to Reduce Immunogenicity

The CTxA1 starting material or a conjugate or fusion protein thereof produced in Example I may be subjected to a controlled chemical modification reaction scheme as shown in Fig. 13. The proteinaceous starting material is incubated with

citraconic anhydride under conditions sufficient to selectively modify easily accessible lysine residues (i.e., 10-50:1 offering citraconic anhydride to protein ratio, pH 9-9.5). The modified protein is then reacted with succinic anhydride at neutral pH and at high offering (excess of succinic anhydride) to modify all other susceptible residues. The protein is then dialyzed in phosphate buffer at pH 5.0, allowing hydrolysis of the lysine adducts and restoration of the original epsilon amino functionality. Modification control and extensive succinylation are achieved using this immunogenicity reducing method, while the BRM activity of the conjugate, dependent on lysines, is retained.

EXAMPLE III

Immunogenicity Testing

Pseudomonas exotoxin A, for example, is very immunogenic in both mice and humans, and both types of recipients respond to the same immunodominant site(s) of the toxin molecule (Morgan, unpublished information). To examine the immunogenicity of unmodified vs. succinylated PE, BALB/c mice were injected with 1 μg/mouse NR-LU-10/PE conjugate (unmodified), NR-LU-10-PE conjugate (toxin was succinylated prior to conjugation) or HSA. Anti-PE titers were determined by solid phase ELISA at days 0, 14 and 28. NR-LU-10 is an antibody that recognizes a 37-40 kd pan-carcinoma glycoprotein.

Mice injected with NR-LU-10-PE conjugate demonstrated a high titer anti-PE response at day 14 post-injection (Table IX). Control mice (HSA administered) showed no anti-PE response.

TABLE IX

Immunogenicity of Chemically Modified Pseudomonas Exotoxin A Conjugates in Individual BALB/c Mice

Control	Native Toxin	Chemically Modified Toxin
31.68*	1688.72	52.48
65.18	704.54	161.32
64.79	1283.46	19.01
65.81	2029.58	159.94
53.03	1337.88	77.81

* Anti-toxin titers at day 14 post-injection.

As shown in Table IX, succinylation of PE significantly reduces the immunogenicity thereof in mice. In a like manner, immunogenicity of other bacterial toxins may be reduced by succinylation. Chemical modification of PE does not markedly affect ADP-ribosylating activity (data not shown).

EXAMPLE IV

Inhibition of Tumor Cell Proliferation

A conjugate of epidermal growth factor (EGF) and an adenylate cyclase toxin portion derived from B. anthraces is prepared in accordance with previously described recombinant procedures. Generally, a patient bearing a tumor which is EGF positive will receive a single, daily pharmacologically effective dose of the conjugate by an intravenous administration route. When the conjugate is delivered to tumor cells, the adenylate cyclase toxin is internalized therein. This BRM is expected to catalyze the conversion of ATP to cAMP. Upon the increase in intracellular cAMP levels, proliferation of the tumor cells will be inhibited and/or the cells will be caused to differentiate, resulting in tumor stasis.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

Claims

1. A non-cytolytic toxin moiety useful for altering a functional process of a target cell to achieve a therapeutic objective, the moiety comprising:

a non-cytolytic toxin portion comprising a holotoxin or a portion of a holotoxin having enzymatic activity having a predominant effect that is not a direct inhibition of protein synthesis; and

a targeting moiety selective for target cells and capable of preferentially delivering the conjugate to the target cell or signaling a desired biological response.

2. A non-cytolytic toxin moiety according to claim 1 wherein the non-cytolytic toxin portion comprises an enzymatic toxin portion capable of eliciting a target cell response through the operation of a target cell signaling mechanism and the targeting moiety comprises a ligand involved in the target cell signaling mechanism.

3. A non-cytolytic toxin moiety according to claim 1 wherein the non-cytolytic toxin portion affects a G-protein or modified G-protein modulated signaling mechanism operating within the target cell.

4. A non-cytolytic toxin moiety according to claim 3 wherein the G-protein signaling process regulates at least one effector molecule within the target cells.

5. A non-cytolytic toxin moiety according to claim 4 wherein the effector molecule is selected from the group comprising adenyl cyclase,

phosphodiesterase, calcium channels, and cAMP dependent protein kinase.

6. A non-cytolytic toxin moiety according to claim 4 wherein the non-cytolytic toxin portion catalyzes ADP ribosylation of a G alpha s substrate.

7. A non-cytolytic toxin moiety according to claim 4 wherein the non-cytolytic toxin portion catalyzes ADP ribosylation of a G alpha i or G alpha o.

8. A non-cytolytic toxin moiety according to claim 2 wherein the non-cytolytic toxin portion is derived from, isolated from or corresponds to a toxin selected from the group comprising cholera toxin, pertussis toxin, E. coli enterotoxin, botulinum neurotoxin, botulinum C2 toxin, and clostridial iota toxin.

9. A non-cytolytic toxin moiety according to claim 8 wherein the non-cytolytic toxin portion comprises an enzymatic A1 portion of cholera toxin.

10. A non-cytolytic toxin moiety according to claim 8 wherein the non-cytolytic toxin portion comprises an enzymatic S1 portion of pertussis toxin.

11. A non-cytolytic toxin moiety according to claim 1 wherein the non-cytolytic toxin portion is capable of inducing a biological response in the target cells independent of target cell metabolic signaling mechanisms.

12. A non-cytolytic toxin moiety according to claim 11 wherein the non-cytolytic toxin portion modulates target cell cAMP level.

13. A non-cytolytic toxin moiety according to claim 12 wherein the non-cytolytic toxin portion comprises an enzymatic portion of a bacterial or mammalian adenylate cyclase enzyme.

14. A non-cytolytic toxin moiety according to claim 13 wherein the non-cytolytic toxin portion comprises an enzymatic portion of an adenylate cyclase toxin of Bordetella or Bacillus origin.

15. A non-cytolytic toxin moiety according to claim 1 wherein the non-cytolytic toxin portion is capable of modifying actin.

16. A non-cytolytic toxin moiety according to claim 1 wherein the targeting moiety comprises a growth factor, a hormone, antibody, a cytokine, or a colony stimulating factor.

17. A non-cytolytic toxin moiety according to claim 1 wherein the non-cytolytic toxin portion does not exhibit endogenous cell binding activity.

18. A non-cytolytic toxin moiety according to claim 1 wherein the non-cytolytic toxin portion comprises a functionally nullified endogenous cell binding portion.

19. A non-cytolytic toxin moiety according to claim 1 wherein the non-cytolytic toxin portion or moiety has been chemically or recombinantly altered to decrease the protease sensitivity thereof.

20. A non-cytolytic toxin moiety according to claim 1 wherein the non-cytolytic toxin portion or moiety has been chemically or genetically altered to decrease the immunogenicity thereof.

21. A non-cytolytic toxin moiety according to claim 1 wherein the non-cytolytic toxin portion and targeting moiety are bound through a conditionally unstable peptide linker.

22. A non-cytolytic toxin moiety according to claim 21 wherein the linker comprises a consensus endosome protease sequence sensitive to endosomal and lysomal enzymes and contained within a disulfide loop.

23. A non-cytolytic toxin moiety of claim 1 wherein the non-cytolytic toxin portion comprises cholera toxin A1 subunit or a functional equivalent thereof and the targeting moiety comprises interleukin-2.

24. A non-cytolytic toxin moiety of claim 1 wherein the non-cytolytic toxin portion comprises pertussis toxin S1 subunit or a functional equivalent thereof and the targeting moiety comprises interleukin-7.

25. A non-cytolytic toxin moiety of claim 1 wherein the non-cytolytic toxin portion comprises B. pertussis toxin enzymatic portion or a functional equivalent thereof and the targeting moiety comprises epidermal growth factor, transforming growth factor (TGF) alpha or beta, transferrin, bombesin, gastrin releasing peptide.

26. A method of inducing an in vivo or ex vivo biological response in a target cell population, the method comprising administration to a patient or in vitro incubation of a sample containing the target cell population of a non-cytolytic toxin moiety which comprises:

a non-cytolytic toxin portion comprising a holotoxin or a portion of a holotoxin having enzymatic activity, having a predominant effect that is not a direct inhibition of protein synthesis; and

a targeting moiety selective for target cells and capable of preferentially delivering the conjugate to the target cell or eliciting a desired biological response.

27. A method according to claim 26 wherein the target cell population comprises bone marrow stem cells and the biological response comprises proliferation.

28. A method according to claim 27 wherein the targeting moiety is administered in combination with an antiviral (HIV) replication inhibitory agent or agent to inhibit HIV infection.

29. A method according to claim 28 wherein the HIV inhibiting agent comprises AZT, BI-RG-587, or CD-4 peptides or functional equivalents thereof.

30. A method according to claim 26 wherein the target cell population comprises activated T-cells and the biological response comprises inhibition of target cell proliferation to achieve prolongation of organ allografts.

31. A method according to claim 26 wherein the target cell population comprises tumor cells and the biological response comprises modulation of target cell cAMP level.

32. A method according to claim 31 wherein an increase in target cell cAMP level results in target cell differentiation, thereby increasing target cell expression of surface antigens or receptors.

33. A method according to claim 32 wherein the target cell surface antigen being up-regulated is a tumor-associated antigen, thereby enhancing the target cell response to subsequent therapy mediated by the tumor-associated antigen.

34. A method according to claim 32 wherein the target cell surface antigen being up-regulated is a hormone receptor, thereby enhancing the target cell response or susceptibility to subsequent hormonal therapy.

35. A method according to claim 26 wherein the target cell population is CD-5 positive B cells and the biological response comprises a decrease in proliferation or in auto-immune immunoglobulin, thereby decreasing symptomology associated with auto-immune diseases.

36. A method according to claim 26 wherein the target cell population comprises tumor cells and is screened <u>in vitro</u> for sensitivity to multiple retargeted toxin moieties to determine an efficacious treatment protocol by locating and exploiting a functional signaling mechanism in the majority or individual tumors.

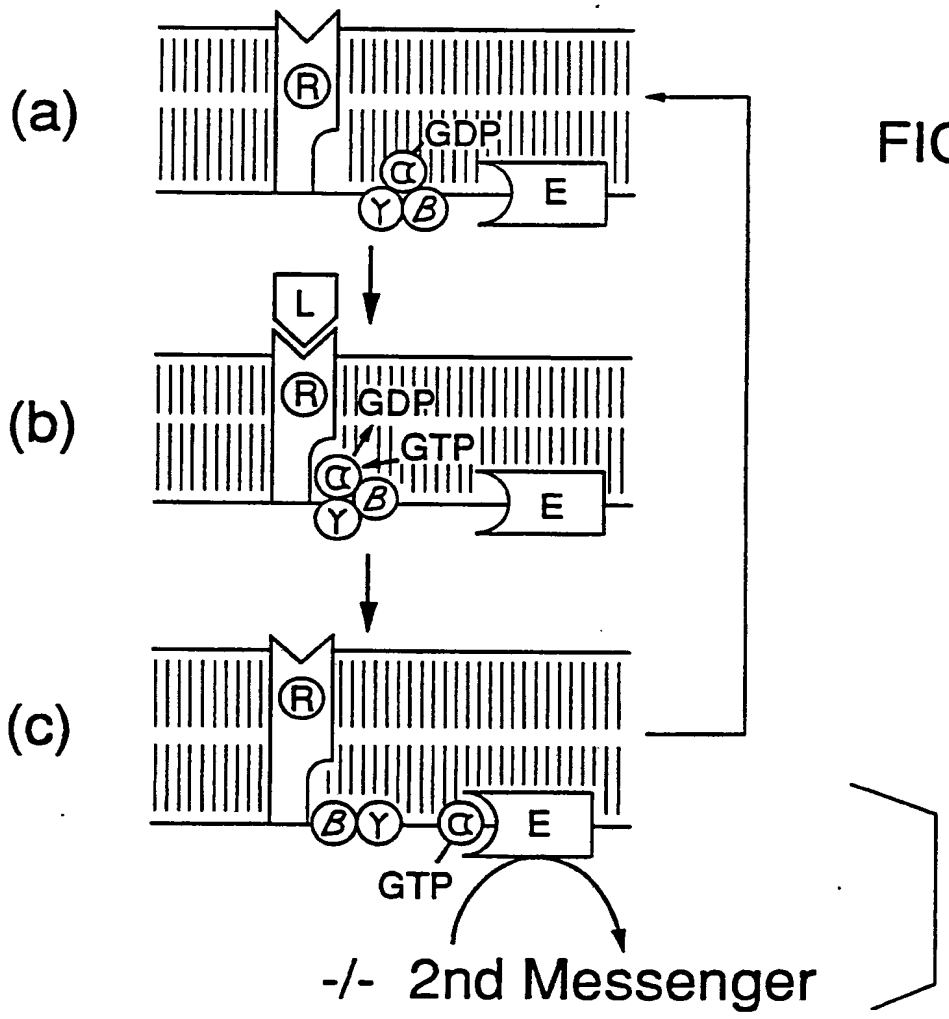


FIG. 1

FIG. 2a

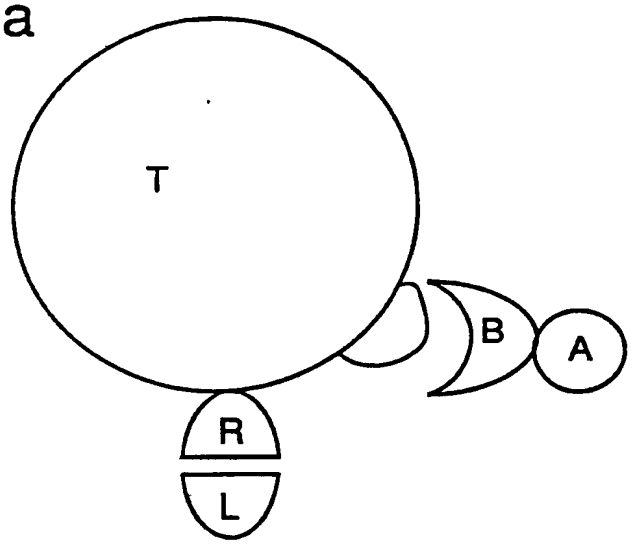


FIG. 2b

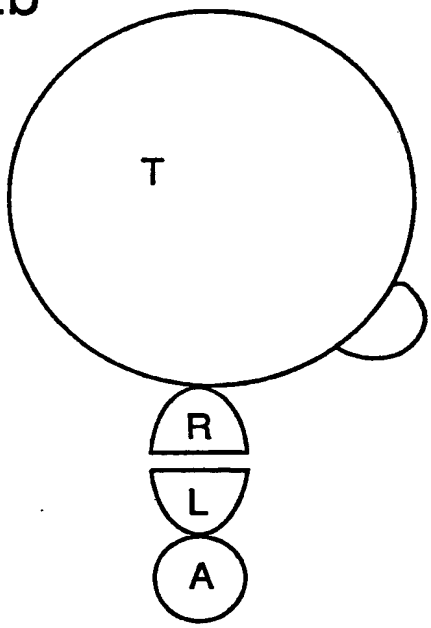


FIG. 3

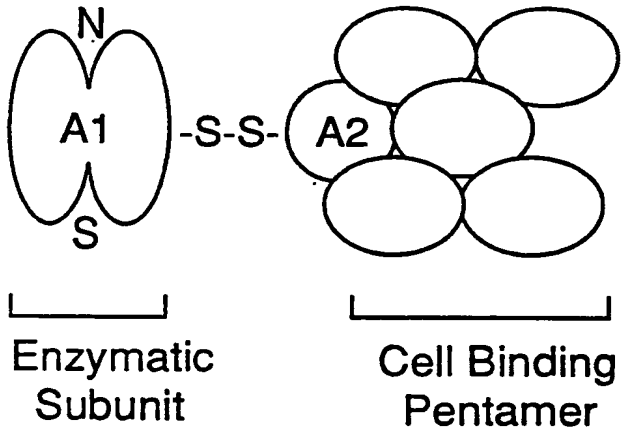

N
A1
S
-S-S-
A2
Enzymatic Subunit
Cell Binding Pentamer

FIG. 4

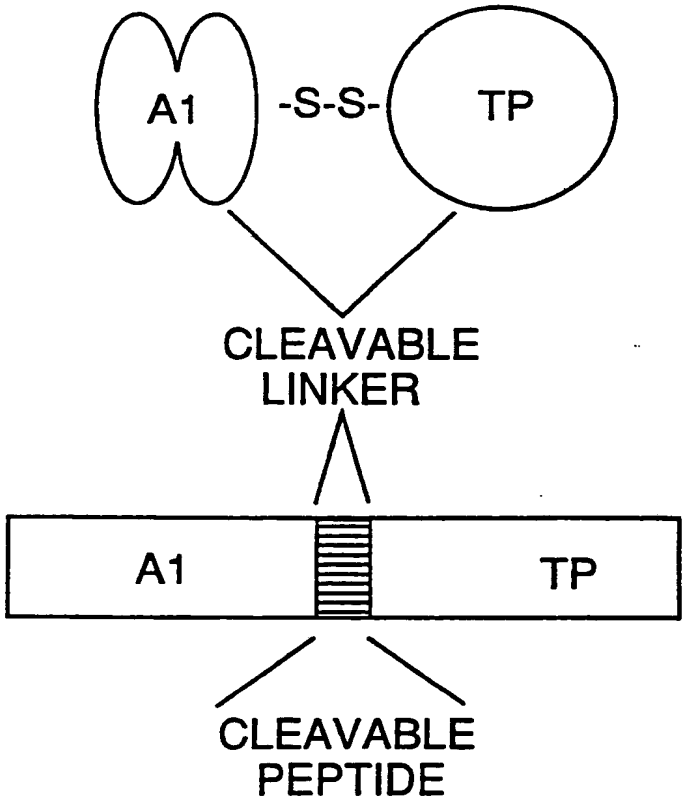

A1
-S-S-
TP
CLEAVABLE LINKER
A1
TP
CLEAVABLE PEPTIDE

FIG. 5

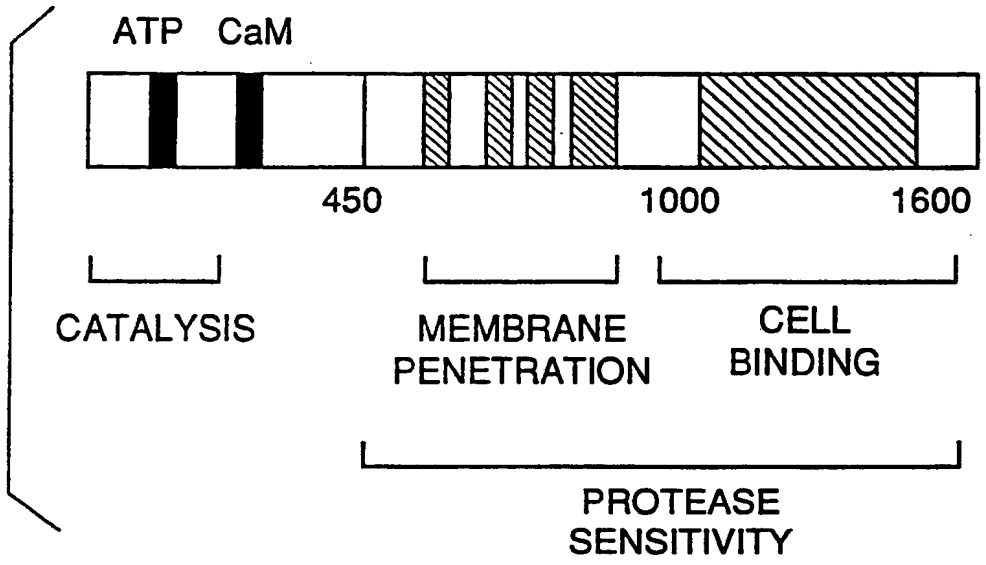

ATP
CaM
450
1000
1600
CATALYSIS
MEMBRANE PENETRATION
CELL BINDING
PROTEASE SENSITIVITY

FIG. 6

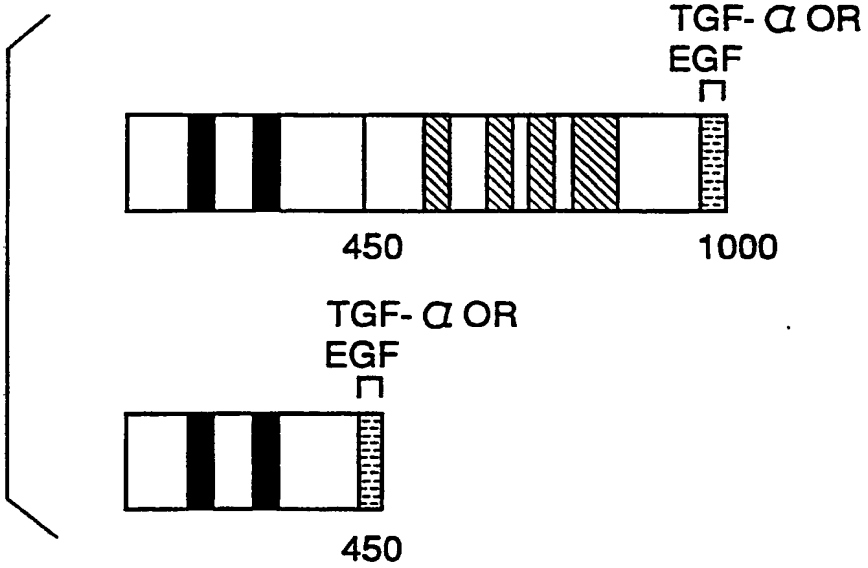

TGF- α OR EGF
450
1000
TGF- α OR EGF
450

FIG. 7

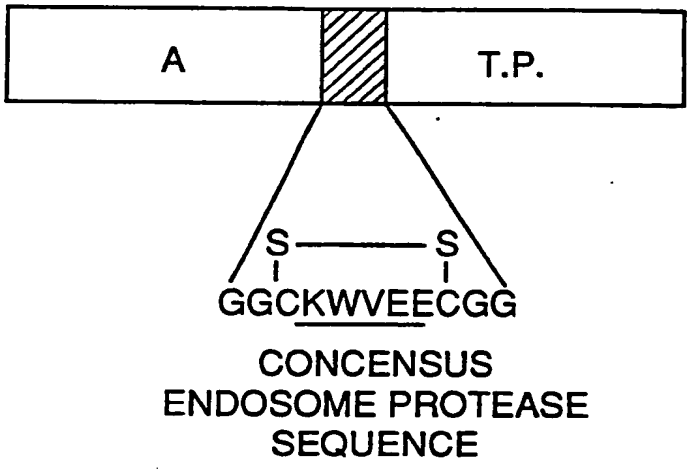

A
T.P.
S
S
GGCKWVEECGG
CONCENSUS
ENDOSOME PROTEASE
SEQUENCE

FIG. 8

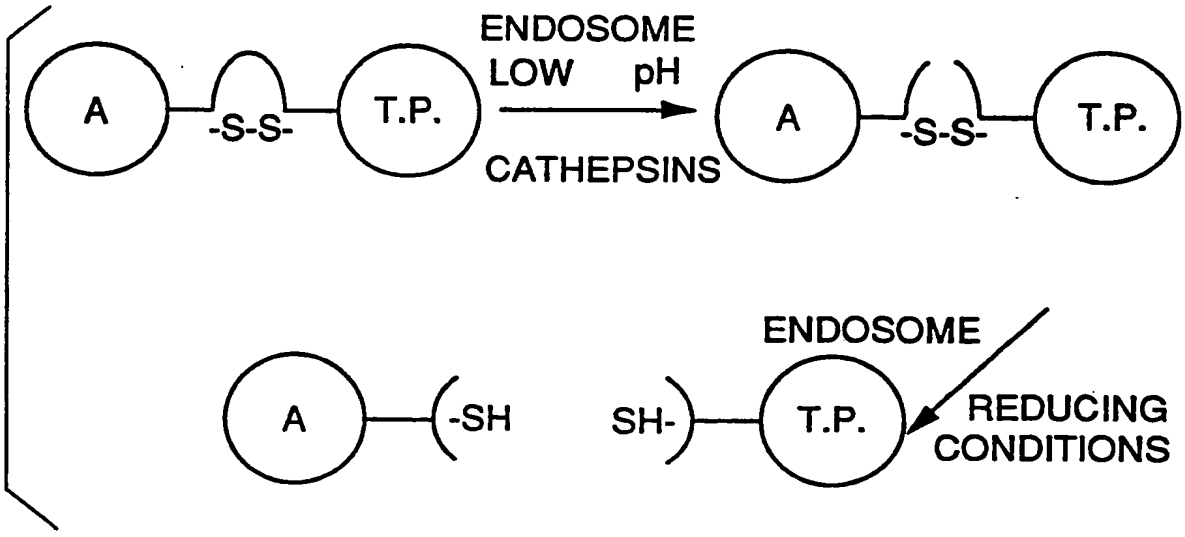

A
-S-S-
T.P.
ENDOSOME
LOW pH
CATHEPSINS
A
-S-S-
T.P.
A
-SH
SH-
T.P.
ENDOSOME
REDUCING
CONDITIONS

FIG. 10

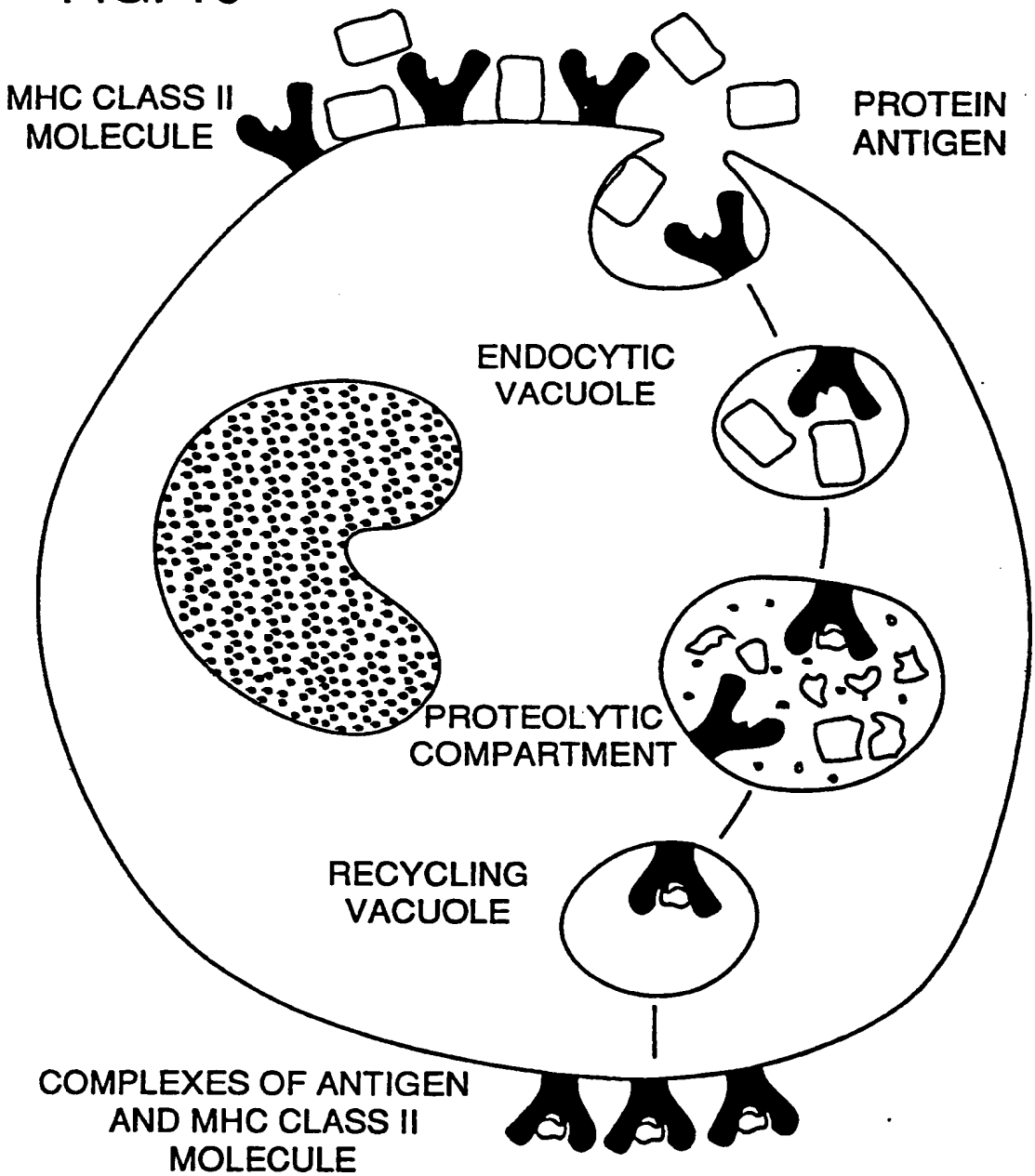

MHC CLASS II MOLECULE
PROTEIN ANTIGEN
ENDOCYTIC VACUOLE
PROTEOLYTIC COMPARTMENT
RECYCLING VACUOLE
COMPLEXES OF ANTIGEN AND MHC CLASS II MOLECULE

FIG. 9

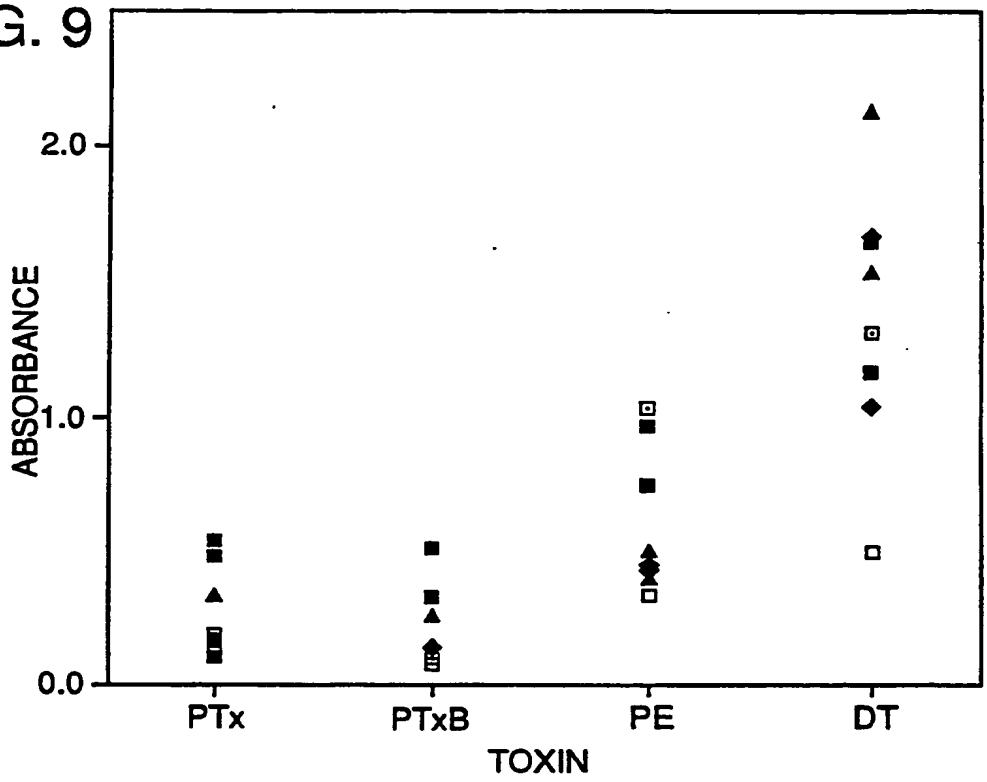
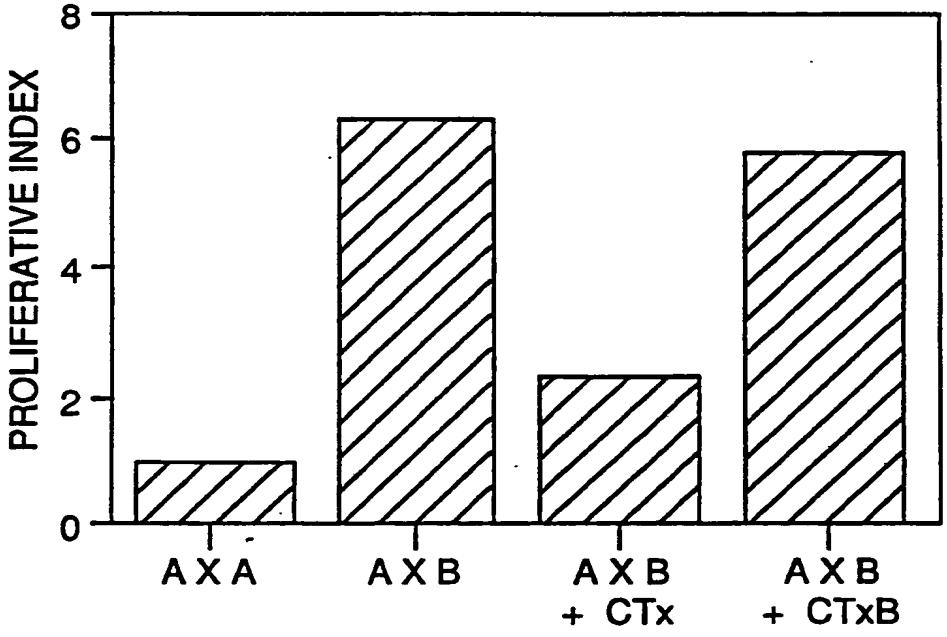

2.0
1.0
0.0
ABSORBANCE
PTx
PTxB
PE
DT
TOXIN

FIG. 11


INHIBITION OF MLR BY CHOLERA TOXIN
8
6
4
2
0
PROLIFERATIVE INDEX
A X A
A X B
A X B + CTx
A X B + CTxB

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/06823

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q, 1/00; G01N, 33/53
US CL :435/7.23
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 188; 424/85.2, 85.8, 85.91, 94.1; 930/240.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,664,911 (UHR ET AL.) 12 MAY 1987, SEE THE ENTIRE DOCUMENT.	1-36
Y	PROC. NATL. ACAD. SCI. USA, VOLUME 83, ISSUED NOVEMBER 1986, MURPHY ET AL., "GENETIC CONSTRUCTION, EXPRESSION, AND MELANOMA-SELECTIVE CYTOTOXICITY OF A DIPHTHERIA TOXIN-RELATED ALPHA-MELANOCYTE-STIMULATING HORMONE FUSION PROTEIN", SEE THE ENTIRE DOCUMENT.	1-36
Y	J. EXP. MED., VOLUME 167, ISSUED FEBRUARY 1988, BACHA ET AL., "INTERLEUKIN 2 RECEPTOR-TARGETED CYTOTOXICITY: INTERLEUKIN 2 RECEPTOR-MEDIATED ACTION OF A DIPHTHERIA TOXIN-RELATED INTERLEUKIN 2 FUSION PROTEIN". PAGES 612-622, SEE THE ENTIRE DOCUMENT.	1-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 19 OCTOBER 1992	Date of mailing of the international search report 24 NOV 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile N . NOT APPLICABLE	Authorized officer GIAN WANG Teleph ne N . (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*